REMARKS/ARGUMENTS

The invention

This invention provides for an anti-allergy peptide comprising a cell-penetrating peptide [CPP] from Kaposi fibroblast growth factor fused to either of two specific inhibitors of mast cell activation. In a test of four different CPPs, the claimed CPP from Kaposi fibroblast growth factor was the *only* CPP that transported its inhibitor domains in a manner that **inhibited** mast cell activation.

Amendments to the specification

As requested by the Examiner, the specification has been amended to set forth the claim to priority and the title has been amended to be more descriptive.

The Examiner requested that the specification be reviewed by spelling errors and improper use of trademarks. A spell check of the document did not reveal any spelling errors and Applicants' attorney did not identify any improper use of trademarks. Applicants respectfully request clarification of this concern.

The Examiner requested that the Applicants review the specification for compliance with the sequence rules. The application was amended to comply with the rules on February 11, 2004. If the Examiner has some specific concerns, she is respectfully requested to identify them and Applicants will comply.

Status of the pending claims

Claims 44, 46, 52-56, 59, 60, 63-70 and 72-80 were pending. Claims 44, 46, 52-56, 59 and 60 are canceled by this amendment. Claims 63-70, 72-76 and 77-80 are pending.

Examiner Interview

Pursuant to Rule 133(b), applicants acknowledge with gratitude the interview of November 2, 2006, with Examiners Crowder and Gambrell. During the interview the rebuttal

evidence for the *prima facie* case of obviousness was presented for comment. No agreement was reached; however, the Examiners acknowledged that experimental evidence of unexpected results was a common means to rebut a *prima facie* case of obviousness. In addition, Examiner Gambrell asked if additional evidence of the unpredictability of bioactivity of peptides transported by cell-penetrating peptides might be available. Upon inquiry, the principle inventor, Ronit Eisenberg was unaware of other reports.

REJECTIONS

35 U.S.C. §112 1ST Paragraph

The pending claims were rejected for reciting the word "preventing". As suggested by the Examiner, the claims now recite "inhibiting" mast cell degranulation.

In addition, the Examiner has rejected the claims as overly broad because Jones *et al.* describes results with the Gi3a carboxy terminal transported by a cell-penetrating peptide [CPP] termed "TP10" and derived from the mast cell secretagogue, mastoparan. Jones reports that their CPP chimeric construct is internalized; but its biological properties were not as predicted. The chimeric peptide activated pathways that lead to mast cell degranulation. More specifically, Jones *et al.* reports that their CPP activated dual phosphorylation in p42/p44 MAP kinases in U373MG atrocyoma cells (nerve cells) and weakly promoted release of beta hexoseaminidase in RBL-2H3 cells. Both activation of MAP kinases and release of β hexoseaminidase are characteristic signals of degranulation in mast cells.[1]

Applicants note that their results are in agreement with Jones *et al.* As will be explained below in the section on §103, the selection of the cell penetrating peptide is critical to functionality of the mast cell inhibitor domain. Of three CPPs tested by Applicants, only the CPP from Kaposi fibroblast growth factor **inhibited** mast cell degranulation.

[1] Zhang *et al.* 1997 JBC 272(20):13397-13402 (attached as **Exhibit 1**) is presented as evidence of the relationship between mast cell degranulation and MAP kinase activation and hexoseaminidase release. See in particular the first paragraph on page 13397 (MAP), and page 13398 at the first sentence of the carryover ¶ between columns 1 and 2.

With the claims appropriately limited to the CPP that is competent to deliver the two mast cell inhibitors in a functional manner, Applicants submit that they have fully complied with the enablement requirements.

35 U.S.C. §103(a)

Holgate in view of Aridor and Lin

The Examiner rejected the pending claims as obvious over Holgate in view of Aridor and Lin. Holgate is relied upon as generally teaching that pharmacological agents can inhibit mast cell degranulation and these agents are useful for treating diseases such as asthma. Aridor teaches Seq. No. 1 (KNNLKECGLY) and Lin teaches the CPP (AAVALLPAVLLALLAP). The Examiner then creates the *prima facie* case of obviousness by arguing that she has identified the salient elements of the claims, a motivation to combine the elements, and a reasonable expectation that once combined, the recited elements would function to inhibit histamine release by mast cells.

Applicants respectfully urge that the last element of the *prima facie* case of obviousness has not been properly set forth. There is no reasonable expectation that once a cargo peptide is transported into a cell by a CPP, the peptide will retain its biological properties. In fact, the Examiner admits in her enablement argument that based on the Jones *et al.* reference, the field is not predictable. Indeed, the Applicants' own work as recorded in the specification supports this conclusion of unpredictability.

More specifically, the record before us provides results for four different CPPs transporting the inhibitors of mast cell activation. Three by the Applicants' work and one from Jones *et al.* Only the claimed CPP functioned as desired. One could not find a clearer example of rebutting a *prima facie* case of obviousness.

Applicants agree with the Examiner that there is a reasonable expectation that any CPP would transport any cargo across a cell. It is the retention of biological function of the cargo peptide that is unpredictable. On page 10 of the specification, there are 6 peptides numbered 1-6. Each peptide represents a combination of three different CPPs and two mast cell activation

inhibitor domains. The CPPs are human integrin β3 [Hu Int], Kaposi fibroblast growth factor [KFGF] and the homeodomain of a Drosophila transcription factor [Dros].[2] The two mast cell activation inhibitors are $G\alpha i_3$ and Gαt.

The table below summarizes Applicants' results as described in the specification and the Jones's work.

CHIMERIC PEPTIDE			RESULTS
Hu Int	$G\alpha i_3$	SEQ ID NO: 6	No inhibition of histamine secretion
KFGF	$G\alpha i_3$	SEQ ID NO: 7	**Inhibited histamine secretion**
Dros	$G\alpha i_3$	SEQ ID NO: 10	Induced histamine secretion
Hu Int	Gαt	SEQ ID NO: 11	No inhibition of histamine secretion
KFGF SEQ ID NO: 3	Gαt	SEQ ID NO: 12	**Inhibited histamine secretion**
Dros	Gαt	SEQ ID NO: 13	Induced histamine secretion
TP-10	$G\alpha i_3$	Jones *et al.*	Induced histamine secretion

From Applicants' results and the confirming prior art of Jones *et al.*, it is clear that there is no *a priori* expectation that a particular CPP can both: a) transport $G\alpha i_3$ and Gαt peptides; ***and***, b) retain their inhibitory effects on mast cell degranulation. For this reason, the *prima facie* case of obviousness is fully rebutted. Unless the Examiner can articulate objective reasons that the field is predictable, the rejection of the pending claims as obvious must be withdrawn.

[2] The human integrin β3 CPP is described in Hawiger, 1997 Current Opinion in Immunology 9:189-194 (attached as **Exhibit 2**): see page 189, 2nd col describing both Hu Int and the KFGF as CPPs. The *Drosophila* CPP is described in Derossi *et al.* 1996 JBC 271(30):18188-18193 (attached as **Exhibit 3**): see first sentence of abstract.

During the interview, Examiner Chun asked the undersigned attorney to explain the impact of Aridor's results. It was explained that Aridor by itself merely taught that the anti-allergy peptides [AAP] have biological activity. There is no mention of CPP technology. In the subject application, the claims read on a combination of CPP and AAP and that combination needs to be the focus of the *prima facie* case of obviousness. Here the fact that only one of four tested CPP's maintains the biological activity is the basis for rebutting the *prima facie* case of obviousness.

Holgate in view of Aridor and Lin and further in view of Avruch and Jackson.

Claims 64 and 65 are rejected under 35 U.S.C. §103(a) over Holgate in view of Aridor and Lin and further in view of Avruch and Jackson. Holgate, Aridor and Lin are relied upon as set forth above. Avruch describes the value of succinylation of the amino terminal ends of peptides to facilitate cell penetration and Jackson teaches the value of cyclization of a peptide to stabilize confirmation. Claims 64 and 65 depend from claim 63, and Applicants rely upon the above arguments for claim 63 to rebut the *prima facie* case of obviousness.

Double Patenting

The pending claims are provisionally rejected on the grounds of double patenting over claims 1-44 of copending USSN 10/465,826 and claims 1-15 of copending USSN 11/214,588. No substantive response is deemed helpful at this time. According to the MPEP at page 804, a provisional double patenting rejection must be withdrawn in one of the applications when it is the sole remaining basis for rejection.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

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Mitogen-activated Protein (MAP) Kinase Regulates Production of Tumor Necrosis Factor-α and Release of Arachidonic Acid in Mast Cells

INDICATIONS OF COMMUNICATION BETWEEN p38 AND p42 MAP KINASES*

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Aggregation of the high affinity IgE receptor (FcεRI) in a mast cell line resulted in activation of the p42 and the stress-activated p38 mitogen-activated protein (MAP) kinases. Selective inhibition of these respective kinases with PD 098059 and SB 203580 indicated that p42 MAP kinase, but not p38 MAP kinase, contributed to the production of the cytokine, tumor necrosis factor-α, and the release of arachidonic acid in these cells. Neither kinase, however, was essential for FcεRI-mediated degranulation or constitutive production of tumor growth factor-β. Studies with SB 203580 and the p38 MAP kinase activator anisomycin also revealed that p38 MAP kinase negatively regulated activation of p42 MAP kinase and the responses mediated by this kinase.

Stimulation of mast cells by aggregation of membrane IgE receptors (FcεRI), leads to recruitment of the tyrosine kinase Syk and activation of Syk-dependent signaling cascades (1, 2). These cascades include activation of phospholipase C and sphingosine kinase for mobilization of calcium ions and PKC[1] (3, 4) and the activation of p42 MAP kinase cascade through Ras (2, 5). These cascades lead ultimately to secretion of intracellular granules, a response primarily driven by the increase in $[Ca^{2+}]_i$ and activation of PKC (6), and a $cPLA_2$-mediated release of arachidonic acid. The activation of $cPLA_2$ is dependent on increase of $[Ca^{2+}]_i$ and phosphorylation by MAP kinase (2, 7, 8).

Stimulated mast cells also produce a variety of cytokines that include interleukins 1, 3, 4, 5, and 6 as well as TNFα and granulocyte-macrophage colony-stimulating factor (9, 10). Typically, increased expression of cytokine mRNA and protein is detectable 30 min to several hours after the addition of stimulant (11). These cytokines, particularly TNFα, are thought to mediate pathologic inflammatory reactions (10) and protective responses to bacterial infection (12). The production and release of TNFα are regulated through signals transduced by calcium and PKC, although there are indications that additional FcεRI-mediated signals may operate for optimal production of TNFα in cultured RBL-2H3 mast cells. Compared with antigen, other stimulants are relatively weak inducers of TNFα production when doses of stimulants are matched for maximal stimulation of degranulation (13). Also, concentrations of Ro31–7549 that block PKC, secretion of granules, and release of TNFα only partially block production of TNFα (13).

The present objective was to determine whether stimulation of MAP kinases induces additional signals for production of TNFα. A linkage between these events has not been established in mast cells. Antigen-induced stimulation of p42 MAP kinase coincides with the activation of its upstream regulators, Ras, Raf, and MEK-1 (2, 5), and persists through the period when production of TNFα would be most apparent (14). As noted in this paper, however, RBL-2H3 cells also possess the mammalian homologue of the yeast HOG-1 protein kinase, p38 MAP kinase. We have utilized the MEK-1 inhibitor, PD 098059 (15, 16), and the p38 MAP kinase inhibitor, SB 203580 (17), to evaluate the role of these MAP kinases in the production of TNFα and, for comparison, the release of arachidonic acid, degranulation, and production of TGFβ. Release of arachidonic acid is thought to be dependent on phosphorylation of $cPLA_2$ by MAP kinase, although the identity of the MAP kinase is uncertain (18). Degranulation and TGFβ production were assumed to be MAP-kinase-independent responses (7, 19). We show that, while p42 MAP kinase regulated production of both TNFα and arachidonic acid, p38 MAP kinase negatively regulated the activation of p42 MAP kinase and the responses mediated by this kinase.

MATERIALS AND METHODS

Reagents—Reagents were obtained from the following sources: all reagents for cell culture from Life Technologies, Inc.; ATP from Boehringer Mannheim; adenosine 5′-[γ-^{32}P]triphosphate tetra(triethyl-ammonium) salt and [^{14}C]arachidonic acid from DuPont NEN; phenyl-Sepharose from Pharmacia (Uppsala, Sweden); MAP kinase substrates (myelin basic protein and a myelin basic peptide, residues 94–102), polyclonal antibodies against the COOH-terminal peptide of rat MAP kinase R2 (erk1-CT), anti-phosphotyrosine monoclonal antibody, 4G10, and p42 MAP kinase glutathione *S*-transferase fusion protein from Upstate Biotechnology Inc. (Lake Placid, NY); polyclonal antibodies against p38 MAP kinase, MEK, and $cPLA_2$ from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); TGFβ 1 kit and Factor-test™ Mouse TNF-α from Genzyme Corp. (Cambridge, MA). Ro31–7549 was obtained from LC Laboratories. The compounds PD 098059 and SB 203580 were synthesized in the Tsukuba Research Laboratories, Eisai Co., according to the procedures of Bridges *et al.* (20) and Adams *et al.* (21), respectively, and purified by column chromatography and recrystallization. These compounds were determined to be >95% pure on the basis of high

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[1] The abbreviations used are: PKC, protein kinase C; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; MAP, mitogen-activated protein; FcεRI, high affinity receptor for IgE; DNP-BSA, antigen consisting of 24 molecules of *O*-dinitrophenol conjugated with one molecule of bovine serum albumin; TNFα and -β, tumor necrosis factor-α and -β, respectively; TGFβ, transforming growth factor-β; PIPES, 1,4-piperazinediethanesulfonic acid; MOPS, 4-morpholinepropanesulfonic acid; MEK, MAP kinase kinase.

EXHIBIT 1

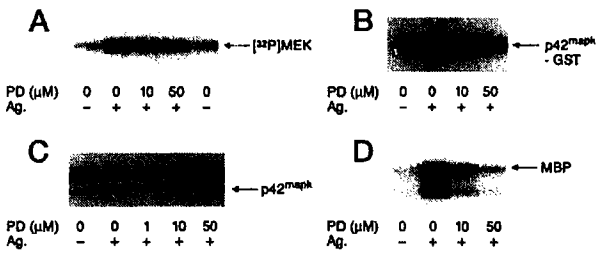

FIG. 1. **Suppression of phosphorylation and activation of MEK and MAP kinase by PD 098059.** For *all panels*, RBL-2H3 cell cultures were left unstimulated (*Ag.*, −) or stimulated with 200 ng/ml DNP-BSA (*Ag.*, +) for 5 min in the presence of the indicated concentrations of PD 098059. *A*, cultures were labeled with [γ-^{32}P]pyrophosphate and then stimulated as described above. MEK was immunoprecipitated from cell lysates and separated by electrophoresis for autoradiography. *B*, unlabeled cultures were stimulated as noted above. MEK was immunoprecipitated and then assayed by phosphorylation of p42^{mapk}-glutathione *S*-transferase fusion protein (extracellular signal-regulated kinase) as substrate with [γ-^{32}P]ATP. ^{32}P-Labeled substrate was separated by electrophoresis for detection by autoradiography as described under "Materials and Methods." *C*, cell lysates were analyzed by Western blotting for p42 MAP kinase. The *lower band*, previously identified as p42 MAP kinase (p42^{mapk}), is indicated. The additional retarded band represents the activated tyrosine-phosphorylated form of p42 MAP kinase (2, 14). *D*, unlabeled cells were stimulated as described above. MAP kinase was immunoprecipitated with antibody against MAP kinase R2 and assayed for MAP kinase activity using myelin basic protein (*MBP*) as the substrate. Phosphorylated protein was separated by electrophoresis and detected by autoradiography.

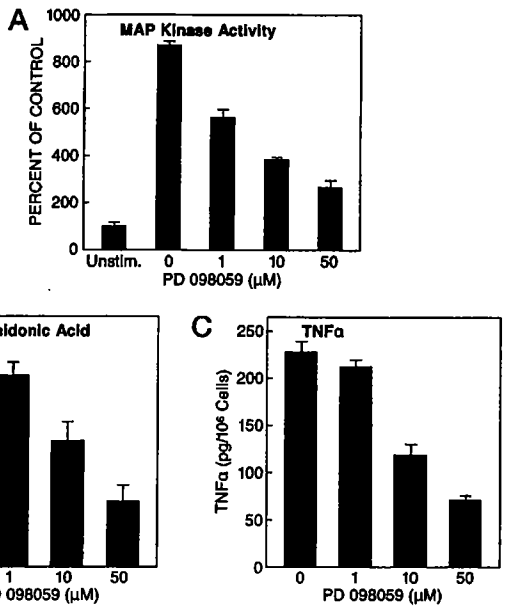

FIG. 2. **Suppression of MAP kinase-dependent responses by PD 098059.** *A*, cultures were incubated with the indicated concentrations of PD 098059 for 30 min and then either left unstimulated (*Unstim.*) or stimulated for 5 min with 20 ng/ml DNP-BSA. MAP kinase activity was assayed in whole cell extracts as described under "Materials and Methods." *B*, cultures were incubated with the indicated concentrations of PD 098059 for 30 min and then stimulated with 20 ng/ml DNP-BSA for 15 min for measurement of release of arachidonic acid. *C*, cultures were incubated for 10 min with PD098059 and then stimulated for 135 min with 20 ng/ml DNP-BSA for measurement of production of TNFα (*C*) as described under "Materials and Methods." The values for this and all other figures were mean (±S.E.) values from three separate experiments (two cultures in each).

performance liquid chromatography and NMR analysis. The antigen, DNP-BSA, and *O*-dinitrophenol-specific monoclonal IgE were kindly supplied by Dr. Henry Metzger (NIAMS, National Institutes of Health, Bethesda, MD).

Cell Culture and Measurement of Stimulatory Responses—The RBL-2H3 cell line was maintained in complete growth medium (minimum essential medium) supplemented with 15% fetal calf serum, glutamine, antibiotic, and antimycotic agents. Trypsinized cells were plated into 150-mm culture dishes or six-well Costar cluster plates and were incubated overnight in complete growth medium with *O*-dinitrophenol-specific IgE (0.5 μg/ml) and, for measurement of arachidonic acid release, [^{14}C]arachidonic acid (0.1 μCi/ml).

Cultures were washed the next day and replenished with the required medium. For the assay of hexosaminidase or [^{14}C]arachidonic acid, experiments were performed in a PIPES-buffered medium (25 mM PIPES, pH 7.2, 159 mM NaCl, 5 mM KCl, 0.4 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.6 mM glucose, and 0.1% fatty acid-free fraction V bovine serum albumin). For [^{32}P]phosphorylation of proteins, cultures were incubated for 90 min with ^{32}P-labeled orthophosphate in PIPES-buffered medium exactly as described (7). For all other assays, experiments were performed in complete growth medium supplemented with 15% fetal calf serum (for measurement of TNFα), 5% fetal calf serum (for measurement of TGFβ), or 0.1% bovine serum albumin and 25 mM Hepes, pH 7.2 (for assay of MAP kinases and separation of proteins by immunoprecipitation and electrophoresis). The inhibitors were added either 30 min (PD 098059) or 15 min (SB 203580 and indomethacin) before stimulation of cultures with antigen (DNP-BSA) as described in the figure legends.

Measurement of Degranulation, Release of [^{14}C]Arachidonic Acid, and Production of TNFα and TGFβ—Release of the granule marker, hexosaminidase, was determined by colorimetric assay of medium and cell lysates by previously described procedures (6). For measurement of release of arachidonic acid, cells were labeled to equilibrium with [^{14}C]arachidonic acid before the addition of inhibitors and antigen as described above. Reactions were terminated by placing cultures on ice and rapidly removing medium. The medium was briefly centrifuged (Beckman Microfuge for 30 s) to remove extraneous cells. Both medium and cell lysates (in 0.1% Triton X-100) were assayed for hexosaminidase (6) and radiolabel (22). Values were expressed as the percentage of intracellular hexosaminidase or radiolabel that was released into the external medium, and they were corrected for spontaneous release from unstimulated cells. It should be noted that, in RBL-2H3 cells, arachidonic acid is metabolized in part to leukotriene C_4/B_4 and prostaglandin D_2 via the 5-lipoxygenase and cyclooxygenase pathways, respectively (23, 24). Release of radiolabel, as measured in this paper, was an estimate of total release of [^{14}C]arachidonic acid and its metabolites. The cytokines were assayed as described elsewhere (19). Whole cell lysates were prepared by freezing and thawing the cultures three times. TGFβ was assayed with a human TGFβ enzyme-linked immunosorbent assay kit, which utilized a mouse monoclonal anti-human antibody that cross-reacted with rat TGFβ. TNFα was assayed with a murine TNFα enzyme-linked immunosorbent assay kit, which utilized a monoclonal hamster anti-murine antibody that reacted with mouse or rat TNFα and -β. The limits of detection for these assays were 25 pg of TGFβ/10^6 cells and 6 pg of TNFα/10^6 cells. Values were corrected for spontaneous release in the absence of stimulant (≤3% for release of hexosaminidase, ≤2% for release of arachidonic acid, and undetectable release of TNFα) except for TGFβ, which was produced constitutively in RBL-2H3 cells (19).

Assay of MAP Kinase Activity in Cell Extracts—After stimulation of cultures in six-well cluster plates, the cultures were washed once, and the medium was removed. The plates were then placed on ice before the addition of 510 μl of a Tris buffer (25 mM Tris, pH 7.5, 25 mM NaCl, 1 mM Na_3VO_4, 2 mM EGTA, 1.5 mM dithiothreitol, 2.5 mM *p*-nitrophenyl phosphate, and 20 μg/ml leupeptin and aprotinin). Cells were disrupted by freezing and thawing three times. The lysate was centrifuged (15,800 × *g* for 10 min), and 450 μl of the supernatant fraction was mixed with 50 μl of ethylene glycol and 80 μl of washed phenyl-Sepharose. The phenyl-Sepharose was washed beforehand with 300 μl of the Tris buffer. The mixture was kept on ice for 5 min for binding of MAP kinase to the beads. After centrifugation, the phenyl-Sepharose beads were washed with 1 ml of 10% (v/v) ethylene glycol and then with 30% (v/v) ethylene glycol. Finally, MAP kinase was eluted by incubating the beads with 75 μl of 60% ethylene glycol for 5 min on ice. After centrifugation of the suspension, 15 μl of supernatant was incubated (15 min, 30 °C) in a solution that contained 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, [γ-^{32}P]ATP (10 Ci/mmol, 37 kBq/tube), and 25 μg of MAP kinase substrate peptide (peptide 94–102 of bovine myelin basic protein). The phosphorylated peptide was isolated by centrifugation of the incubation mixture through phosphocellulose membrane (SpinZyme; Pierce), which was then washed twice with 500 μl of 75 mM H_3PO_4 for the assay of radioactivity.

Assay of MAP Kinase and MEK Activities by Immunoprecipitation—

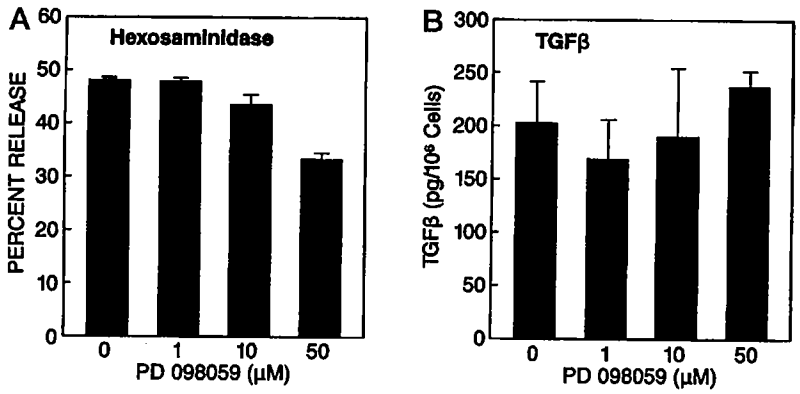

FIG. 3. **Release of hexosaminidase and TGFβ is not inhibited by PD 098059.** *A*, cultures were incubated with the indicated concentrations of PD 098059 for 30 min and then stimulated for 15 min with 20 ng/ml DNP-BSA for measurement of release of hexosaminidase. *B*, cultures incubated with indicated concentrations of PD 098059 for 14 h without stimulation for the assay of TGFβ production as described under "Materials and Methods."

p42 and p38 MAP kinases were immunoprecipitated with the appropriate polyclonal antibodies by procedures described elsewhere (2). Equal amounts of immunoprecipitated proteins from 5×10^6 cells were incubated in a MOPS buffer (25 mM β-glycerol phosphate, 1 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 25 mM MOPS, pH 7.2) with Mg^{2+}-[γ-^{32}P]ATP (10 μCi in 150 μM cold ATP, 25 μM $MgCl_2$) and 5 μM myelin basic protein (18 kDa) as substrate in a final volume of 30 μl. The mixture was incubated at 30 °C for 12 min. The reaction was terminated by the addition of 30 μl of 2 × SDS sample buffer. MEK was immunoprecipitated with anti-MEK antibody and assayed similarly except that p42 MAP kinase glutathione *S*-transferase fusion protein (1 μg/assay) was used as substrate for phosphorylation. Proteins were separated by 12% SDS-PAGE. Radioactive proteins were detected by autoradiography.

Electrophoretic Separation and Immunoblotting of [^{32}P]MEK, p42 MAP Kinase, and cPLA$_2$—The preparation of cell lysates and immunoprecipitates, analysis of proteins by SDS-PAGE, and transfer to nitrocellulose paper were performed as described elsewhere (2, 7) with the following exception: cPLA$_2$ was separated on NOVEX 10% Tris/glycine gels for 3 h at 35 mA and 4 °C as described by Kramer and co-workers (25). Previously described procedures were used for isolation and detection of [^{32}P]MEK (7). Otherwise, proteins were detected by the immunoblotting technique with antibodies against MEK, p42 MAP kinase, cPLA$_2$, or anti-phosphotyrosine. Secondary antibodies included horseradish peroxidase-conjugated antibody against rabbit IgG or mouse IgG. Finally, proteins were visualized by the ECL System (Amersham Corp.) or by autoradiography.

RESULTS

MEK Inhibitor PD 098059 Inhibits the Activity of p42 MAP Kinase, Release of Arachidonic Acid, and Production of TNFα—As shown in Fig. 1, the MEK inhibitor PD 098059 attenuated antigen-induced [^{32}P]phosphorylation of MEK (*panel A*) and the activation of MEK as determined by *in vitro* assay of immunoprecipitated MEK (*panel B*). Activation of p42 MAP kinase was also attenuated, as indicated by the change in electrophoretic migration of p42 MAP kinase (*panel C*) or by the assay of MAP kinase activity of immunoprecipitated p42 MAP kinase (*panel D*). The extent of these inhibitions was dependent on the concentration of PD 098059. As shown in Fig. 2, the suppression of MAP kinase activation by PD 098059 (*panel A*) was associated with similar dose-dependent suppression of arachidonic acid release (*panel B*) and TNFα production (*panel C*). The suppression of the latter two responses was highly correlated ($r > 0.95$). All three responses were inhibited by ~50% with 10 μM PD 098059. As will be described later, activation of cPLA$_2$ was also inhibited by PD 098059. These results suggested that release of arachidonic acid and production of TNFα were both regulated by p42 MAP kinase.

The Effect of PD 098059 on Degranulation and TGFβ Production—To test the selectivity of PD 098059, we next examined the effects of this compound on antigen-stimulated degranulation and the constitutive production of TGFβ, which are thought not to be regulated by MAP kinase (7, 19). PD 098059 had only minimal effects on stimulated release of the granule constituent, hexosaminidase (Fig. 3*A*) and the production of TGFβ in unstimulated cells (Fig. 3*B*). The only significant effect was partial inhibition (<30%) of degranulation at 50 μM PD 098059 (Fig. 3*A*).

The p38 MAP Kinase Inhibitor, SB 203580, Enhances Activation of p42 MAP Kinase, Release of Arachidonic Acid, and Production of TNFα in Antigen-stimulated Cells—Antigen stimulation also resulted in increased activity of p38 MAP kinase (Fig. 4*A*, compare *lanes 1* and *2*). The p38 kinase inhibitor, SB 203580, inhibited this activation (Fig. 4*A*, *lanes 3* and *4*). Interestingly, antigen activation of p42 MAP kinase was enhanced significantly by SB 203580. This enhancement was apparent when cells were stimulated with 20 or 200 ng/ml antigen (Fig. 4*B*). The latter concentration of antigen was known to elicit maximal activation of p42 MAP kinase.[2] These results suggested that p38 MAP kinase negatively regulates p42 MAP kinase and that this regulation is alleviated by SB 203580.

The enhanced activation of p42 MAP kinase in the presence of SB 203580 was associated with increased release of arachidonic acid (Fig. 5*A*) and production of TNFα (Fig. 5*B*). In the experiment shown in Fig. 5*B*, cells were stimulated with a low concentration of antigen (6 ng/ml) to maximize enhancement of the TNFα response (250% increase in Fig. 5*B*). At optimal doses of antigen enhancement of TNFα production was less (40–80% increase) but still significant (data not shown).

Because pyridinyl imidazoles that are closely related to SB 203580 have cyclooxygenase-inhibitory activity (25, 26), experiments were conducted to determine whether blockade of cyclooxygenase activity with indomethacin (27) altered accumulation of radiolabel in the medium by suppressing metabolism [^{14}C]arachidonate via this enzyme. Unlike SB 203580, 10 μM indomethacin did not significantly alter release of radiolabel from antigen-stimulated cells (7.8 ± 0.4% release over 15 min *versus* 7.2 ± 0.2% release in the absence of indomethacin; mean ± S.E. in eight cultures from two experiments). It seemed probable, therefore, that SB 203580 enhanced release rather than the accumulation of [^{14}C]arachidonic acid in the medium.

In contrast to the increased release of arachidonic acid and production of TNFα, SB 203580 had no significant effect on antigen-induced degranulation (Fig. 5*C*) or constitutive production of TGFβ (Fig. 5*D*). Collectively, these results provided further evidence for the notion that release of arachidonic acid and TNFα production are both regulated by p42 MAP kinase. In addition, the results suggested that p38 MAP kinase negatively modulates these responses through p42 MAP kinase.

The above results suggested that release of arachidonic acid,

[2] C. Zhang, R. A. Baumgartner, K. Yamada, and M. A. Beaven, unpublished data.

The Journal of Biological Chemistry

as well as production of TNFα, was regulated by p42 MAP kinase. As in other systems (25, 28), the phosphorylation of $cPLA_2$ in stimulated RBL-2H3 cells leads to decreased electrophoretic mobility of the enzyme (7). The connection between p42 MAP kinase and the release of arachidonic acid via $cPLA_2$ was further demonstrated by the finding that the antigen-induced retardation of electrophoretic migration of $cPLA_2$ (25) was suppressed by PD 098059 but not by SB 203580 (Fig. 6).

Activation of p38 MAP Kinase by Anisomycin Partially Suppresses Activation of p42 MAP Kinase and Release of Arachidonic Acid—The p38 MAP kinase activator, anisomycin, markedly activated this kinase (Fig. 7*A*) but much less so p42 MAP kinase (Fig. 7*B*). The combination of anisomycin and antigen revealed inhibitory communication between these two kinase. For example, the combination of stimulants resulted in less activation of p38 MAP kinase (Fig. 7*C*, *lane 3*) than that induced by antigen (Fig. 7*C*, *lane 2*) or anisomycin (Fig. 7*A*, *lane 2*) alone. The combination also caused less activation of p42 MAP kinase (Fig. 7*D*, *lane 3*) than that by antigen alone (Fig. 7*D*, *lane 2*). Thus, stimulation of p42 MAP kinase by antigen appeared to block activation of p38 MAP kinase by anisomycin, and conversely stimulation of p38 MAP kinase by anisomycin appeared to partially block activation of p42 MAP kinase by antigen. Consistent with the latter situation, anisomycin partially suppressed antigen-induced release of arachidonic acid (25 ± 4% reduction, mean of three experiments). This reduction corresponded to an approximately 25% reduction in p42 MAP kinase activation as determined by densitometric analysis of the blots shown in Fig. 7*D* and two other experiments. Anisomycin almost totally blocked (by 83 ± 4%) antigen-induced production of TNFα, probably as a consequence, however, of its known inhibitory actions on protein synthesis at the translation step (29). Presumably, *de novo* synthesis of TNFα would be especially sensitive to inhibitors of protein synthesis.

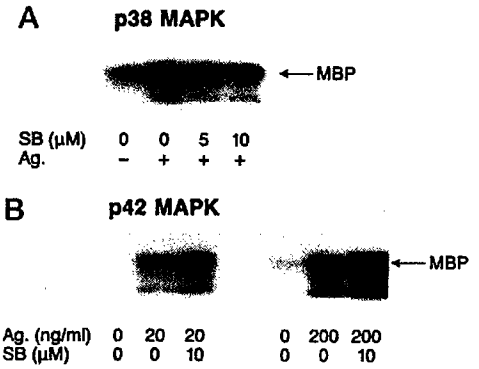

FIG. 4. **The p38 MAP kinase inhibitor, SB203580, suppresses activation of p38 MAP kinase but enhances p42 MAP kinase activity.** Intact RBL-2H3 cells were incubated with the indicated concentrations of SB203580 for 15 min before stimulation with antigen. *A*, cultures were left unstimulated or stimulated with 200 ng/ml DNP-BSA for 4 min before the assay of p38 MAP kinase. *B*, cultures were left unstimulated or stimulated with 20 ng/ml DNP-BSA for 15 min (*left panel*) or with 200 ng/ml DNP-BSA for 4 min (*right panel*) before the assay of p42 MAP kinase. For both panels, the MAP kinases were immunoprecipitated and assayed by use of myelin basic protein (*MBP*) as substrate as described for Fig. 1.

DISCUSSION

Past studies have shown that the responses evoked by antigen in RBL-2H3 cells were dependent on calcium and signals generated through PKC or MAP kinase. These studies indicated, for example, that PKC regulated degranulation (6) as well as the production and secretion of TNFα, although it appeared likely that additional FcεRI-mediated signals facilitated TNFα production (13). Activation of p42 MAP kinase, in contrast, was associated with phosphorylation of $cPLA_2$ and release of arachidonic acid (2, 7). These studies, however, did not address the issue of whether other MAP kinases, such as p38 MAP kinase, regulated $cPLA_2$.

The present results demonstrate that both p38 and p42 MAP kinases are activated in antigen-stimulated cells. Activation of the latter kinase appears to be most closely related to release of arachidonic acid and production of TNFα. All three events are

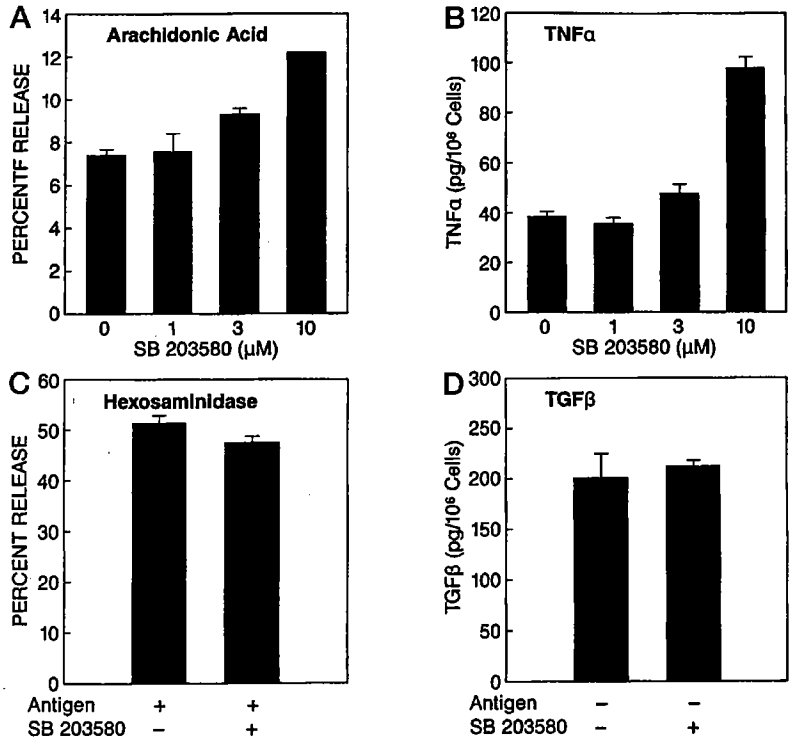

FIG. 5. **Inhibition of p38 MAP kinase leads to the enhanced activation of p42 MAP kinase and production of arachidonic acid and TNFα.** Cultures were incubated without or with SB 203580 (concentrations as indicated or 10 μM for *panels C* and *D*) for 15 min before stimulation with antigen (20 ng/ml DNP-BSA). For *panels A* and *C*, cultures were stimulated for 15 min for the measurement of release of arachidonic acid or hexosaminidase. For *panel B*, cultures were stimulated for 135 min for the measurement of TNFα production, and for *panel D*, cultures were left unstimulated for 14 h for the measurement of TGFβ production.

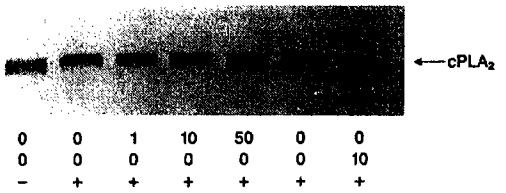

FIG. 6. **Effect of PD 098059 and SB 203580 on antigen-induced retardation of electrophoretic migration of cPLA$_2$.** RBL-2H3 cells were stimulated with 20 ng/ml DNP-BSA (*Ag.*, +) or left unstimulated (*Ag.*, −) for 7 min in the presence or absence of the indicated concentrations of PD 098059 (*PD*) or SB 203580 (*SB*). Proteins in cell lysates were separated by SDS-PAGE, and cPLA$_2$ was detected by probing with anti-cPLA$_2$ antibody as described under "Materials and Methods."

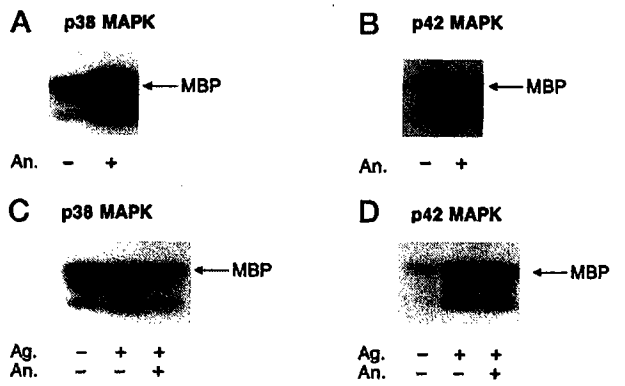

FIG. 7. **Activation of p38 and p42 MAP kinases by anisomycin and antigen.** Intact RBL-2H3 cells were incubated with vehicle (*An.*, −) or 10 μM of anisomycin for 30 min (*An.*, +) and then left unstimulated (*Ag.*, −) or stimulated with 20 ng/ml DNP-BSA (*Ag.*, +) for 5 min before the assay of immunoprecipitated p38 or p42 MAP kinases with myelin basic protein as substrate as described in previous figure legends.

inhibited by the MEK inhibitor, PD 098059 (Fig. 2), and enhanced by the p38 MAP kinase inhibitor, SB 203580 (Figs. 4 and 5). Both compounds are reported to be selective inhibitors of MEK (*i.e.* PD 098059) and p38 MAP kinase (*i.e.* SB 203580) when tested against a wide range of kinases (15–17). In addition, the enhancement of responses in the presence of SB 203580, in contrast to the attenuation of p42 MAP kinase activation by the p38 MAP kinase activator, anisomycin (Fig. 7), suggest that p38 MAP kinase negatively regulates activation of p42 MAP kinase and its associated responses. Antigen-stimulated degranulation and the constitutive production of TGFβ in RBL-2H3 cells are minimally affected by the inhibitors (Figs. 3 and 5). Collectively, the results support the notion that p42 MAP kinase regulates release of arachidonic acid and promotes an additional signal for stimulating TNFα production but does not regulate degranulation. Interestingly, the p38 MAP kinase inhibitor, SB 203580, was first identified as an inhibitor of cytokine biosynthesis in lipopolysaccharide-stimulated human monocytes (17) and was subsequently shown to suppress TNFα production in lipopolysaccharide-injected mice (30). The compound also possessed anti-inflammatory activity in mouse models of arthritis (collagen- and adjuvant-induced), whereas cellular immune responses measured *ex vivo* were unaffected (30). It is possible, therefore, that different MAP kinase pathways are utilized for activating gene transcription for cytokine synthesis when synthesis is induced by inflammatory agents or through multimeric immunologic receptors such as FcεRI.

The question has been raised whether p38 rather than p42 MAP kinase is responsible for the activation of cPLA$_2$ (18, 25). cPLA$_2$ is phosphorylated by both kinases in thrombin-stimulated platelets, although the phosphorylation by p38 MAP kinase does not appear to activate cPLA$_2$ (25). Our results indicate that p42 MAP kinase regulates phosphorylation of cPLA$_2$ and release of arachidonic acid and suggest, therefore, that this kinase is the activator of cPLA$_2$ at least in RBL-2H3 cells.

Mast cells, including RBL-2H3 cells, also contain a low molecular weight secreted form (type II) of PLA$_2$ (31) in secretory granules, and this form is presumably released along with other granule constituents in activated cells (32). A role for secreted PLA$_2$ is unlikely, however, because total suppression of degranulation by selective inhibitors of PKC, such as Ro31–7549 and calphostin C, minimally affects release of arachidonic acid in RBL-2H3 cells (Refs. 7 and 33 and see below).[3] In addition to the correlations between activation of the p42 MAP kinase/cPLA$_2$ pathway and release of arachidonic acid noted here with PD 098059 and SB 203580, similar correlations have been noted in previous studies with less specific MAP kinase inhibitors. Activation of the entire Raf/MEK/p42 MAP kinase pathway and release of arachidonic acid were suppressed equally by the glucorticoid, dexamethasone, and the kinase inhibitor, quercetin, while effects on degranulation were apparent only at relatively high concentrations of these agents (7, 34). Correlations were noted with the PKC inhibitor, Ro31–7549. This inhibitor transiently delayed activation of p42 MAP kinase in antigen-stimulated RBL-2H3 cells. There was a corresponding transient delay in the release of arachidonic acid, although the cumulative release eventually equaled that observed in the absence of Ro31–7549 (14). On the basis of these and other results, we have suggested that the p42 MAP kinase/cPLA$_2$ pathway, although transiently activated by PKC, was predominantly activated by Ras through recruitment of Shc/Grb2/Sos or Vav by FcεRI (14). Others have reported that fatty acids, particularly arachidonic acid, activate p42 and p44 MAP kinases through PKC (35). This scenario is unlikely in antigen-stimulated RBL-2H3 cells, however, because of the predominance of the PKC-independent (*i.e.* Ro31–7549-resistant) pathway in RBL-2H3 cells.

The present data extend previous findings on the regulation of TNFα synthesis and release. This cytokine is synthesized *de novo* and subsequently secreted via Golgi in a PKC- and calcium-dependent manner (13). The PKC inhibitor, Ro31–7549, blocks secretion of TNFα but only partially suppresses synthesis of TNFα (6, 13, 19). Thus, additional signals may be necessary for optimal stimulation of TNFα synthesis. Antigen is a particularly potent stimulant of TNFα production when compared with the combination of calcium ionophore and PKC agonist, phorbol 12-myristate 13-acetate (13, 19). These observations and the present studies with the MAP kinase inhibitors suggest that optimal production of TNFα is achieved through activation of both PKC and p42 MAP kinase.

The present findings may explain why antigen-induced activation of p42 MAP kinase (34), release of arachidonic acid (22, 34) and production of TNFα (36) exhibit similar sensitivity to dexamethasone. All three responses are totally suppressed in RBL-2H3 cells that have been treated with 10 nM dexamethasone, whereas antigen-stimulated hydrolysis of phosphoinositides, increase in $[Ca^{2+}]_i$, and degranulation (22, 34) are only partially suppressed by treatment of cells with 100 nM dexamethasone. Dexamethasone, as noted above, inhibits the entire

[3] In addition to the cytosolic (cPLA$_2$) and secreted (sPLA$_2$) PLA$_2$, a calcium-independent form of PLA$_2$ (iPLA$_2$) has been described in myocytes and smooth muscle cells (40). iPLA$_2$ is inhibited by bromoenol lactone (41) and activated by depletion of intracellular calcium stores or by the calmodulin inhibitor, W7 (40). By use of the same experimental strategies, we find no evidence for the presence of iPLA$_2$ in RBL-2H3 cells (T. Hundley and M. A. Beaven, unpublished data).

Raf/MEK/p42 MAP kinase/cPLA$_2$ pathway at nanomolar concentrations (34). Therefore, if p42 MAP kinase is the common regulator of TNFα production and arachidonic acid release, the similar sensitivities to dexamethasone would be expected.

The connections between the p42 MAP kinase pathway and cytokine production are unknown for mast cells, but recent reports indicate the following connections in other types of cells. The overexpression of Raf1 (37, 38) or p42 MAP kinase (39) results in enhanced expression of a variety of cytokine genes in T cells and macrophages (37, 39), the inactivation of IκB (38), and the enhanced binding activity of cytokine transcription factors such as NF-κB and AP-1 (39).

In conclusion, the above results provide the first indication that p42 MAP kinase regulates antigen-mediated production of TNFα in a mast cell line and that p38 MAP kinase may negatively regulate the p42 MAP kinase/cytokine pathway. We can, for the first time, broadly define the regulatory pathways for all three functional responses of mast cells to antigen as follows. Along with elevated $[Ca^{2+}]_i$, the additional primary signals are as follows: for degranulation (6) and secretion of newly formed TNFα (13), activation of PKC (6); for cPLA$_2$-mediated release of arachidonic acid, activation of p42 MAP kinase (Refs. 2, 7, and 34, and this paper); and for production of TNFα, the coactivation of PKC and p42 MAP kinase (Ref. 13 and this paper).

REFERENCES

1. Scharenberg, A. M., Lin, S., Cuenod, B., Yamamura, H., and Kinet, J. P. (1995) *EMBO. J.* **14,** 3385–3394
2. Hirasawa, N., Scharenberg, A., Yamamura, H., Beaven, M. A., and Kinet, J.-P. (1995) *J. Biol. Chem.* **270,** 10960–10967
3. Choi, O. H., Kim, J.-H., and Kinet, J.-P. (1996) *Nature* **380,** 634–636
4. Beaven, M. A. (1996) *Curr. Biol.* **6,** 798–801
5. Jabril-Cuenod, B., Zhang, C., Scharenberg, A. M., Paolini, R., Numerof, R., Beaven, M. A., and Kinet, J.-P. (1996) *J. Biol. Chem.* **271,** 16268–16272
6. Ozawa, K., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M., Mischak, H., Mushinski, J. F., and Beaven, M. A. (1993) *J. Biol. Chem.* **268,** 1749–1756
7. Hirasawa, N., Santini, F., and Beaven, M. A. (1995) *J. Immunol.* **154,** 5391–5402
8. Nakatani, Y., Murakami, M., Kudo, I., and Inoue, K. (1994) *J. Immunol.* **153,** 796–803
9. Baumgartner, R. A., and Beaven, M. A. (1996) in *Handbook of Experimental Immunology*, 5th Ed. (Herzenberg, L. A., Herzenberg, L., Weir, D. M., and Blackwell, C., eds) Vol. 4, pp. 213.1–213.8, Blackwell Science, Inc., Cambridge, MA
10. Galli, S. J. (1993) *N. Eng. J. Med.* **328,** 257–265
11. Gordon, J. R., Burd, P. R., and Galli, S. J. (1990) *Immunol. Today* **11,** 458–464
12. Galli, S. J., and Wershil, B. K. (1996) *Nature* **381,** 21–22
13. Baumgartner, R. A., Yamada, K., Deramo, V. A., and Beaven, M. A. (1994) *J. Immunol.* **153,** 2609–2617
14. Zhang, C., Hirasawa, N., and Beaven, M. A. (1997) *J. Immunol.*, 158, in press
15. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7686–7689
16. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270,** 27489–27494
17. Lee, J. C., Laydon, J. T., McDonnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Landvatter, S. W., Stickler, J. E., McLaughlin, M. M., Siemens, I. R., Fisher, S. M., Livi, G. P., Adams, J. L., and Young, P. R. (1994) *Nature* **372,** 739–746
18. Kramer, R. M., Roberts, E. F., Strifler, B. A., and Johnstone, E. M. (1995) *J. Biol. Chem.* **270,** 27395–27398
19. Baumgartner, R. A., Deramo, V. A., and Beaven, M. A. (1996) *J. Immunol.* **157,** 4087–4093
20. Bridge, A. J., and Saltiel, A. R. (1995) U. S. Patent 5,525,625
21. Adams, J. L. (1993) International Patent Application WO 93/14081
22. Collado-Escobar, D., Cunha-Melo, J. R., and Beaven, M. A. (1990) *J. Immunol.* **144,** 244–250
23. McGivney, A., Morita, Y., Crews, F. T., Hirata, F., Axelrod, J., and Siraganian, R. P. (1981) *Arch. Biochem. Biophys.* **212,** 572–580
24. Igarashi, Y., Lundgren, J. D., Shelhamer, J. H., Kaliner, M. A., and White, M. V. (1993) *Immunopharmacology* **25,** 131–144
25. Kramer, R. M., Roberts, E. F., Um, S. L., Borsch-Haubold, A. G., Watson, S. P., Fisher, M. J., and Jakubowski, J. A.(1996) *J. Biol. Chem.* **271,** 27723–27729
26. Lee, J. C., Badger, A. M., Griswold, D. E., Dunnington, D., Truneh, A., Votta, B., White, J. R., Young, P. R., and Bender, P. E. (1993) *Ann. N. Y. Acad. Sci.* **696,** 149–170
27. Vane, J. R. (1971) *Nat. New. Biol.* **231,** 232–235
28. Lin, L. L., Wartmann, M., Lin, A. Y., Knopf, J. L., Seth, A., and Davis, R. J. (1993) *Cell* **72,** 269–278
29. Vazquez, D. (1979) *Mol. Biol. Biochem. Biophys.* **130,** 513–527
30. Badger, A. M., Bradbeer, J. N., Votta, B., Lee, J. C., Adams, J. L., and Griswold, D. E. (1996) *J. Pharmacol. Exp. Ther.* **279,** 1453–1461
31. Murakami, M., Kudo, I., Umeda, M., Matsuzawa, A., Takeda, M., Komada, M., Fujimoi, Y., Takahashi, K., and Inoue, K. (1992) *J. Biochem. (Tokyo)* **111,** 175–181
32. Chock, S. P., Schmauder-Chock, E. A., Cordella-Miele, E., Miele, L., and Mukherjee, A. B. (1994) *Biochem. J.* **300,** 619–622
33. Yamada, K., Jelsema, C. L., and Beaven, M. A. (1992) *J. Immunol.* **149,** 1031–1037
34. Rider, L. G., Hirasawa, N., Santini, F., and Beaven, M. A. (1996) *J. Immunol.* **157,** 2374–2380
35. Rao, G. N., Baas, A. S., Glasgow, W. C., Eling, T. E., Runge, M. S., and Alexander, R. W. (1994) *J. Biol. Chem.* **269,** 32586–32591
36. Baumgartner, R. A., Hirasawa, N., Ozawa, K., Gusovsky, F., and Beaven, M. A. (1996) *J. Immunol.* **157,** 1625–1629
37. Reimann, T., Buscher, D., Hipskind, R. A., Krautwald, S., Lohmann-Matthes, M. L., and Baccarini, M. (1994) *J. Immunol.* **153,** 5740–5749
38. Li, S., and Sedivy, J. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9247–9251
39. Park, J. H., and Levitt, L. (1993) *Blood* **82,** 2470–2477
40. Wolf, M. J., Wang, J., Turk, J., and Gross, R. W. (1997) *J. Biol. Chem.* **272,** 1522–1526
41. Ackermann, E. J., Conde-Frieboes, K., and Dennis, E. A. (1995) *J. Biol. Chem.* **270,** 445–450

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Cellular import of functional peptides to block intracellular signaling

Jacek Hawiger

During the past few years, new approaches to the delivery of functional peptides to cells have been developed to probe intracellular protein–protein interactions. These approaches include a method based on the cell membrane permeability properties of the hydrophobic region of the signal sequence. This method provides easy and rapid delivery of functional peptides to a wide spectrum of cells involved in inflammatory and immune reactions (monocytes, endothelial cells, and T lymphocytes) as well as to NIH 3T3 cells and erythroleukemia HEL cells. The method has been applied to block signaling to the nucleus by transcription factors nuclear factor-κB, AP-1, and nuclear factor of activated T cells, and to inhibit cell adhesion regulated by the cytoplasmic tails of integrins β_3 and β_1. New methods of peptide delivery provide direct access to intracellular proteins involved in adhesion, signaling, and trafficking to the nucleus.

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Abbreviations

CARD	cell adhesion regulatory domain
CPPI	cell-permeable peptide import
EGF	epidermal growth factor
FGF	fibroblast growth factor
HEL	human erythroleukemia
LPS	lipopolysaccharide
MPS	membrane permeable sequence
NF	nuclear factor
NFAT	NF of activated T cells
NLS	nuclear localization signal
PMA	4β-phorbol 12-myristate 13-acetate
TNF	tumor necrosis factor

Introduction

Signal transduction, gene transcription, and intracellular protein trafficking constitute fundamental processes in the cell life-cycle. They depend on intracellular protein–protein and protein–DNA interactions utilizing more or less defined domains or consensus sequences. Structure–function analysis of these intracellular proteins can be aided by synthetic peptide analogs representing putative sites of intracellular protein–protein interactions. Unfortunately, the cell membrane is usually impermeable to synthetic peptides that represent structural segments of intracellular proteins. Likewise, their analysis is hampered by the inability of sequence-specific antibodies to penetrate the plasma membrane. Therefore, the microinjection of antibodies and peptides to individual cells or the use of membrane-disrupting pore-forming reagents [1] are invasive techniques usually employed to allow introduction of synthetic peptides or other noncell membrane-permeable molecules into cells. Alternatively, transfection experiments can be used to introduce DNA encoding truncated or mutated intracellular proteins [2,3]. Although such approaches provide valuable sources of information, their inherent limitations hinder progress in structure–function analysis of intracellular proteins.

To overcome these limitations and to facilitate large-scale delivery of functional peptides to intact cells, new noninvasive approaches were undertaken. One of these, called cell-permeable peptide import (CPPI), was based on the selection of the hydrophobic region (h region) of the signal peptide sequence as a 'carrier' for cellular import of relevant segments or motifs of intracellular proteins [4••]. Other noninvasive approaches were based on *Antennapedia* homeodomain 16 residue peptide able to translocate through the membrane of neuronal cells expressing α-2, 8 polysialic acid [5,6•], myristoylated peptides [7], and the Tat protein of HIV-1 [8].

In this review only some of the features of cellular import of functional peptides based on the h region of signal peptides will be discussed and the details of *Antennapedia* homeodomain-based import to neuronal type cells will not be covered. This article can be said to have a biased perspective because the major focus is on applications of cellular import of functional peptides, primarily to hemopoietic cells involved in inflammatory and immune responses.

CPPI based on signal sequence h region

CPPI based on the signal sequence h region has taken advantage of its ability to translocate across cellular membranes. This region, made up of 16 residues in Kaposi fibroblast growth factor (FGF) signal sequence or 15 residues in integrin β_3 signal sequence, was selected as the membrane permeable sequence (MPS) [4••,9••]. The amino-terminal positively charged N region or the carboxy-terminal cleavage site for signal peptidase (C region) were not included in designing MPS. The functional cargo can be attached to the carboxy-terminus of the MPS. If necessary, an epitope tag can also be included to track the presence of the imported peptides in intracellular compartments.

The intracellular localization of imported peptides was verified by a number of criteria (inaccessibility to extracel-

lular proteases, confocal laser scanning microscopy using monospecific anti-peptide antibodies and functionality of imported peptides designed to inhibit intracellular protein–protein interactions; [4••,9••,10•]). The functional effect of imported peptides is sequence-specific, that is imported peptides carrying wild-type sequence motifs of intracellular protein are functional whereas those carrying mutated sequences are not [4••,9••,10•]. Peptide import is rapid, reaching maximum concentration within 45 minutes at 37°C. The intracellular concentration of imported peptide reaches 4% of the peptide added to the media, as determined by counting cell-associated ^{125}I-labeled peptides or by anti-peptide antibodies in cell enzyme-linked immunosorbent assay. The imported peptide is detectable in cells up to 180 minutes after peptide addition ([4••,9••]; TR Torgerson, A Colosia, J Donahue, YZ Lin, J Hawiger, unpublished data).

Mechanism of membrane translocation of signal sequence containing polypeptides

The ability of signal peptides to insert into membranes and their *in vivo* function correlate with the residue-average hydrophobicity of their hydrophobic cores. This is the critical characteristic of signal sequences even though they lack primary sequence identity [11]. Neither the positively charged N region (amino-terminal segment) of the signal sequence nor the C region (signal peptidase cleavage site) is necessary for membrane translocation of preprolactin [12].

It is not presently known whether the characteristics of membrane translocation involved in the export of proteins through the endoplasmic reticulum are applicable to the mechanism of peptide import into the cell mediated by the h region of a signal peptide—a focus of this review. First, we established that although cell-permeable peptide import is mediated by the h region, it is h region nonspecific because synthetic peptides containing the h region from distinct protein signal sequences (Kaposi FGF and human integrin β_3 subunit) are plasma membrane translocation competent [4••,9••]. Second, cell-permeable peptide import is not limited to a particular cell type because it is readily accomplished in seven human and animal cell lines (murine NIH 3T3 cells and endothelial LE-II cells as well as in human monocytic THP-1 cells, endothelial ECV 304 cells, erythroleukemia HEL cells, fibroblasts, and Jurkat T lymphocytes ([4••,9••,10•]; TR Torgerson, A Colosia, J Donahue, YZ Lin, J Hawiger, unpublished data). Third, cellular import of a nonfunctional peptide [4••] seemed to be ATP-independent because it was observed in NIH 3T3 cells depleted of their metabolic ATP pool [4••]. Fourth, the cellular import of synthetic peptides is temperature-dependent and requires the intact architecture of the plasma membrane because treatment with glutaraldehyde prevents peptide import [4••]. Precisely how membrane fluidity and the lateral mobility of membrane proteins influence cellular import of synthetic peptides remains to be determined. Moreover, cellular import of signal sequence-based peptides seems to be independent of caveolae because it is not impaired in T lymphocytes lacking these plasma membrane constituents ([13]; TR Torgerson, A Colosia, J Donahue, YZ Lin, J Hawiger, unpublished data).

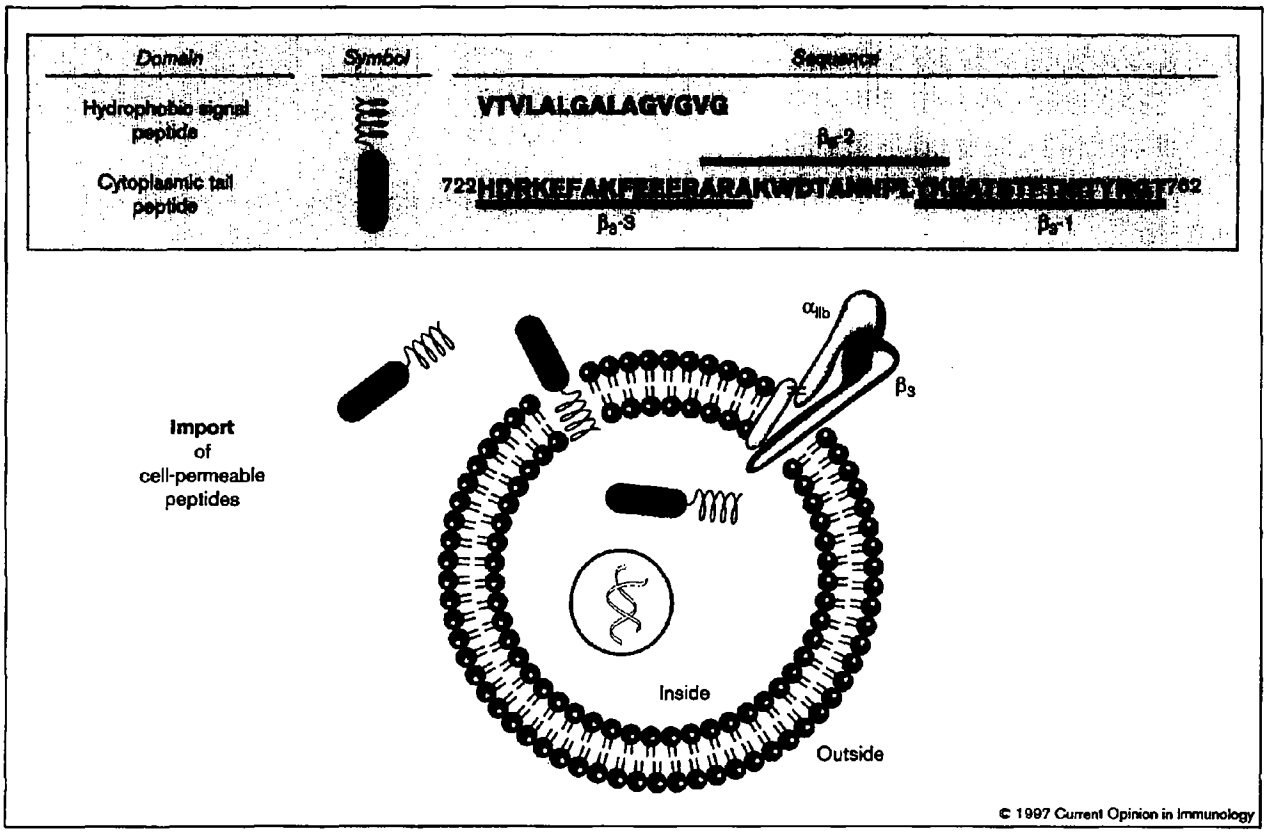
Cellular import of peptides blocking intracellular integrin signaling

Integrins are major two-way signaling receptors responsible for the attachment of cells to the extracellular matrix and for cell–cell interactions which underlie developmental programming, immune responses, tumor metastasis, and progression of atherosclerosis and thrombosis. Whereas the knowledge of the extracellular 'business end' of integrins, responsible for their binding to the ligands and counterreceptors, is relatively well advanced, the intracellular 'business end', engaged in multiple regulatory interactions with signal tranducers and cytoskeletal components, is less understood [14]. The cell-permeable peptide import method was applied to the structure–function analysis of the cytoplasmic tails of integrin $\alpha_{IIb}\beta_3$ which are endogenously expressed in the human erythroleukemia cell line, HEL [9••]. As a functional endpoint, we used adhesion of HEL cells to immobilized fibrinogen in response to stimulation with 4β-phorbol 12-myristate 13-acetate (PMA) [9••]. This adhesion is blocked by integrin $\alpha_{IIb}\beta_3$ polyclonal antibodies. The schematic presentations of this approach to the structure–function analysis of the cytoplasmic tails of integrin $\alpha_{IIb}\beta_3$ subunits is presented below (Fig. 1).

Only cell-permeable peptide β_3-1S, spanning residues 747–762 of the cytoplasmic segment of integrin β_3 ([9••]; Fig. 1), induced the inhibition of adhesion of HEL cells stimulated with PMA (10 nM; Fig. 2). When a membrane translocation-deficient tail peptide, β_3-1, containing sequence 747–762 but lacking a cell membrane permeable sequence (hydrophobic h region of signal sequence) was added, no effect on HEL cellular adhesion was observed (Fig. 2). Accordingly, no fluorescent signal was detectable in HEL cells by confocal laser scanning microscopy (Fig. 3).

When cell-permeable integrin β_3 peptide 747–762 contained a known loss-of-function mutation ($Ser^{752}Pro$; three letter code for amino acids) it was unable to inhibit HEL cell adhesion. An alternative mutation ($Ser^{752}Ala$) we introduced in β_3-1S was silent, suggesting that disruption of the secondary structure of integrin β_3 cytoplasmic tail, rather than the replacement of a potential phosphorylation site, can account for a loss-of-function of the mutant ($Ser^{752}Pro$) peptide. On the other hand, two tyrosine residues 747 and 759 form a functionally active tandem within the cytoplasmic tail of integrin β_3 because conservative mutations $Tyr^{747}Phe$ or $Tyr^{759}Phe$ resulted in a nonfunctional cell-permeable peptide carrying residues 747–763. These structure–function studies using cell-permeable peptide import led to the identification of the

Figure 1



Schematic representations of the cell-permeable peptide import technique as applied to studying the intracellular segments of the integrin $\alpha_{IIb}\beta_3$ complex. Cell-permeable peptides are designed by using the hydrophobic region of the signal peptide of integrin β_3 (represented by the helix-like leading portion) covalently bound to the amino terminus of the selected sequence of the intracellular tail of integrin β_3 (represented by the black ellipse). The sequences employed to construct cell-permeable peptides are shown in the box at the top. β_3-1, β_3-2 and and β_3-3 refer to peptides representing integrin β_3 cytoplasmic sequence [9••].

cell adhesion regulatory domain (CARD) encompassing residues 747–763 in the cytoplasm tail of integrin β_3 that modulates the interaction of integrin $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ with immobilized fibrinogen.

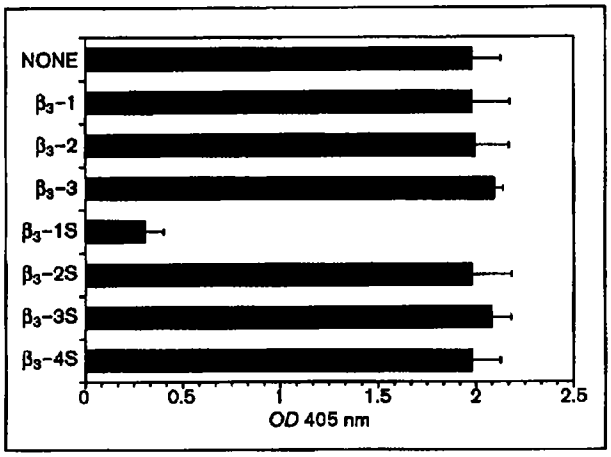
The inhibition of cell adhesion was integrin β_3-specific because a cell-permeable peptide homolog encompassing residues 788–803 of the cytoplasmic tail of integrin β_1 was inactive in an adhesion assay mediated by integrin β_3 (HEL and ECV 304 cells). Conversely, a cell-permeable peptide representing a homologous segment of the integrin β1 cytoplasmic tail (residues 788–803) inhibited adhesion of human fibroblasts mediated by integrin β_1 heterodimers whereas a cell-permeable integrin β_3 peptide (residues 747–762) did not. Thus, an homologous CARD domain is present in the integrin β_1 cytoplasmic tail [9••].

Cellular import of peptides blocking signaling to the nucleus

The nuclear import of many proteins including DNA-binding transcription factors and viral proteins depends on a short peptide sequence called the nuclear localization signal (NLS). Typically, NLS motifs represent a cluster of basic amino acids (lysine and arginine) flanked by acidic residues and/or proline [15,16]. The cytoplasmic protein with an unmasked NLS is recognized by the cytoplasmic NLS receptor with the help of an accessory protein (p90) [17]. A newly-formed complex of protein-NLS receptor-p90 is then bound to the cytoplasmic face of the nuclear pore before the NLS-bearing protein is shuttled to the nucleus. It is clear that the NLS motif serves as a 'zip code' for the nuclear translocation machinery in the cytoplasm. We have demonstrated [4••] effective blockade of this machinery by introducing cell-permeable peptides carrying the NLS motif of the transcription factor nuclear factor (NF)-κB p50 to murine endothelial and human monocytic cell lines stimulated with proinflammatory agonists, lipopolysaccharide (LPS) and tumor necrosis factor (TNF)-α.

A single cell-permeable peptide containing a typical NLS derived from NF-κB p50 blocked inducible nuclear import

Figure 2



Effect of the integrin β_3 cytoplasmic tail peptides on adhesion of HEL cells to immobilized fibrinogen. HEL cells were preincubated without (NONE) or with 200 μM peptides. The bars labeled with β_3-3, β_3-2, and β_3-1 represent adhesion of cells treated with noncell-permeable peptides encompassing the integrin β_3 cytoplasmic sequence containing residues 722–737, 735–750, and 747–762, respectively. The bars labeled with β_3-3S, β_3-2S, β_3-1S, and β_3-4S represent adhesion of cells incubated with the cell-permeable peptides containing the signal sequence hydrophobic region followed by residues 722–737, 735–750, 747–762, and 742–755, respectively. Adhesion was quantitated by a cellular acid phosphatase assay and is expressed as *OD* 405 nm. Data are the mean±standard error of the mean (SEM) from at least three experiments performed in triplicate. The differences in adhesion between control cells preincubated in the absence of peptides and cells treated with β_3-1S peptide are statistically significant at $P \leq 0.002$. Reproduced with permission from [9••].

of NF-κB, AP-1, and nuclear factor of activated T cells (NFAT), factors known to be imported to the nucleus and to be essential for gene transcription in activated T lymphocytes. Inhibition of nuclear import of transcription factors resulted in the attenuation of interleukin-2 gene expression in the human T cell Jurkat line. In addition, expression of IκBα, an inhibitor of NF-κB, was also decreased (TR Torgerson, A Colosia, J Donahue, YZ Lin, J Hawiger, unpublished data). These results indicate that a common mechanism for nuclear import of three distinct families of transcription factors can be functionally inhibited by cell-permeable NLS peptide.

The role of the NLS in the mitogenic activity of FGF-1 in NIH 3T3 cells was also studied using cellular import of functional peptides. This mitogenic effect depends on the presence of a putative NLS encompassing residues 21–27 [18]. Consistent with this report, a cell-permeable peptide carrying the NLS from FGF-1-stimulated DNA synthesis. The effect bypassed FGF-receptor-mediated signaling. A mutant NLS peptide containing two lysine replacements (lysine→threonine) was mitogenically inactive [10•]. Recently, Rojas *et al.* [19•] have applied cell-permeable peptide import to control epidermal growth factor (EGF)-stimulated Ras activation in NIH 3T3 cells overexpressing human EGF receptors [19•]. A cell-permeable peptide carrying one phosphotyrosine-containing segment of the EGF receptor representing its autophosphorylation site (tyrosine 1068) inhibited Ras activation and mitogen-activated protein kinase activity.

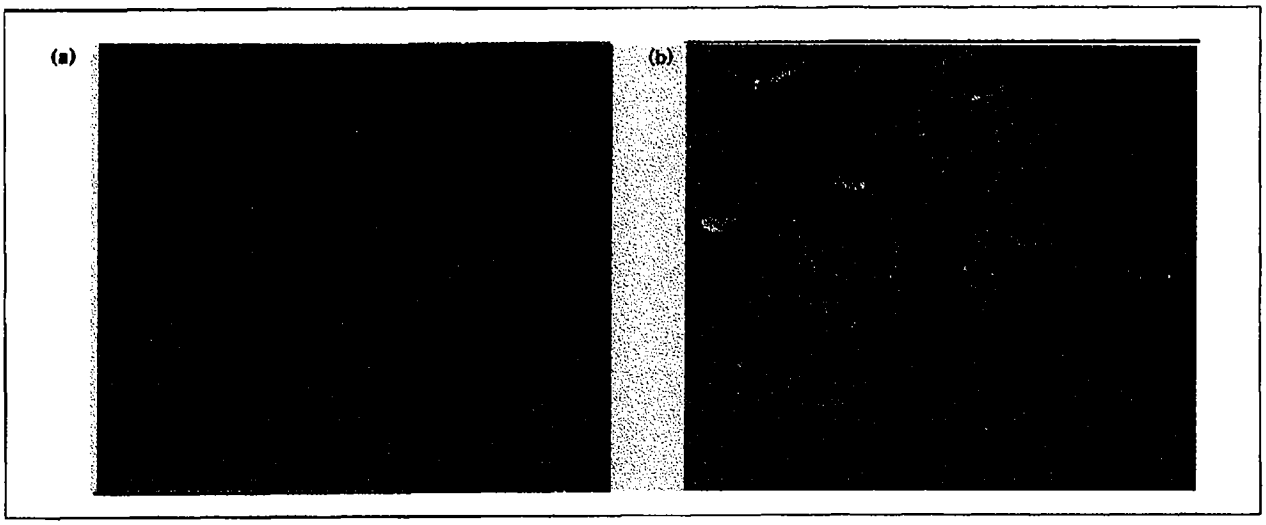
Other ways of importing peptides to cells

A surprising observation that the 60-amino acid homeodomain of *Antennapedia* rapidly crosses the plasma membrane of primary culture of neuronal cells by an energy-independent mechanism gave rise to its application as a nuclear targeting agent. It has a special predilection for neuronal cells expressing neuronal cell adhesion molecule decorated with α-2, 8-polysialic acid which is required for internalization of *Antennapedia* homeobox domain [20]. The varying expression of neuronal cell adhesion molecule in different cell types may contribute to a much lower uptake by non-neuronal cells, such as fibroblasts [21]. The third helix within the homeodomain encompassing 16 residues was shown to translocate through the cell membrane in a temperature-, energy-, and chiral receptor-independent manner [5,6•]. This peptide method was used for intraneuronal delivery of protein kinase C pseudosubstrate [22], Cu/Zn superoxide dismutase antisense oligonucleotide [23], and the peptide inhibitor of the interleukin 1 beta converting enzyme [24].

Another approach to cellular import of peptides takes advantage of amino-terminal myristoylation of peptides representing the pseudosubstrate sequence of protein kinase C [7]. Whereas attachment of a myristoyl group to the amino-terminus provides a suitable membrane 'anchor' for inhibitors of protein kinase C, it potentially restricts the use of this inhibitor to the cytoplasmic face of the plasma membrane. A different approach to cellular import of peptides is based on the synthesis of defined branched peptides ('loligomers') as intracellular vehicles [25]. Unfortunately, these complex molecules exhibited intolerable cytotoxicity, limiting their use for structure–function analysis of intracellular protein in living cells.

A similar concern can be raised in regard to the proposed use of the Tat protein of HIV-1 as a carrier for heterologous protein import into cells [8]. Tat-mediated uptake of beta galactosidase, horseradish peroxidase, RNase A and *Pseudomonas* exotoxin A domain III by endothelial cells and macrophages was documented *in vivo*. However, neither the extent of Tat immunogenicity nor the known biologic effects of Tat *per se* on cells targeted for protein–peptide delivery is clear. Untoward effects of Tat include alteration of normal neuronal and astrocyte organization [26], upregulation of β_2 integrins in monocytes, thereby increasing their adhesiveness [27], angiogenic activities [28], and apoptosis of cultured human blood mononuclear cells [29].

Figure 3



Intracellular location of cell-permeable integrin β_3-1S peptide as demonstrated by confocal laser scanning microscopy (midcell 1 μm section). Intracellular peptide was detected as yellow stains by an indirect immunofluorescence assay and analyzed by a six-step Z-position sectional scanning of the cell. **(a)** Minimal staining of HEL cells treated with non cell-permeable β_3-1 peptide. **(b)** HEL cells treated with cell-permeable β_3-1S peptide clearly show a gain in fluorescent signal representing peptide in the cytoplasm of the HEL cells. A similar pattern was obtained with cells treated with the cell-permeable β_3-2S and β_3-3S peptides (not shown). The anti-peptide β_3-1 antibody used for detection of cell-permeable β_3-1S peptide was monospecific. Reproduced with permission from [9••].

Conclusions

Signal transduction pathways involving intracellular domains of receptors such as integrins, receptor kinases, nonreceptor kinases, intracellular proteases, and transcription factors can be effectively probed using noninvasive cellular import of functional peptides. In some situations, the known functional domains, for example nuclear localization sequences, can be imported to block translocation of transcription factors to the nucleus. In other situations, the cellular import of peptides provides the opportunity to conduct a detailed structure–function analysis of cytoplasmic segments of integrins and other receptors. Peptide mimetics of functionally relevant motifs can be used to block intracellular protein–protein interactions. The utility of noninvasive cell-permeable peptide import based on the hydrophobic region of signal sequence has been firmly established and offers a vast array of applications in probing and blocking intracellular protein–protein and protein–DNA interactions. A wide range of cell types, the speed and ease of translocation across the plasma membrane, free movement to cytoplasmic target proteins, low immunogenicity, and easy detectability of cell-permeable peptides overcome the inherent limitations of currently used invasive methods such as microinjection of individual cells or the use of membrane permeabilizing reagents.

Undoubtedly, these characteristics will be enhanced as the result of ongoing studies on the fundamental mechanism of membrane translocation, subcellular distribution and turnover, and potential cytotoxity of functional peptides. Such insights will shed light on the mechanism of all attempted noninvasive methods of delivery of bioactive molecules into living cells. This assessment is also needed before their usefulness in cell culture experiment can be expanded to the whole organism. Nevertheless, using noninvasive cellular import of bioactive molecules for their selective *in vivo* delivery to different types of cells is within the realm of possibility.

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I would like to thank my colleagues whose collaborative efforts contributed to the recent experimental work discussed in this review: Ann Colosia, John Donahue, Yao Zhong Lin, Xue-Yan Liu, Sheila Timmons, Troy Torgerson, Ruth Ann Veach, and Song Yi Yao. It was supported by the National Institutes of Health NHLBI Merit Award HL-30647 and grants HL 45994 and HL 30648. The assistance of Traci Hill and Carol Walter in the preparation of this manuscript is also acknowledged. The author apologizes to all those whose work has been omitted from the reference list and is instead cited through reviews due to space limitations.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. **Wang K, Feramisco JR, Ash JF: Fluorescent localization of contractile proteins in tissue culture cells.** *Methods Enzymol* 1982, 85:514–562.

2. **Henkel T, Zabel U, Van Zee K, Muller JM, Fanning E, Baeuerle PA: Intramolecular masking of the nuclear location signal and dimerization domain in the precursor for the p50 NF-kappa B subunit.** *Cell* 1992, 68:1121–1133.

3. **Perlmutter RM, Alberola-Ila J: The use of dominant-negative mutations to elucidate signal transduction pathways in lymphocytes.** *Curr Opin Immunol* 1996, 8:285–290.

4. •• Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J: **Inhibition of nuclear translocation of transcription factor NF- kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence.** *J Biol Chem* 1995, 270:14255–14258.

This paper demonstrates the ability of cell-permeable peptides to block signaling to the nucleus by NF-κB, an inflammatory stress-responding transcription factor, in monocytes and endothelial cells stimulated with TNF and LPS.

5. Derossi D, Joliot AH, Chassaing G, Prochiantz A: **The third helix of the Antennapedia homeodomain translocates through biological membranes.** *J Biol Chem* 1994, 269:10444–10450.

6. • Derossi D, Calvet S, Trembleau A, Brunissen A, Chassaing G, Prochiantz A: **Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent.** *J Biol Chem* 1996, 271:18188–18193.

This paper provides evidence that a 16-amino acid peptide corresponding to the third helix of the DNA binding domain (homeodomain) of *Antennapedia*, a *Drosophila* transcription factor, is imported to the cultured embryonic rat brain cells in chiral receptor-independent mode because reversal of sequence or use of D-enantiomers did not prevent cell import at 4°C and 37°C.

7. Eichholtz T, De Bont DB, De Widt J, Liskamp RM, Ploegh HL: **A myristoylated pseudosubstrate peptide, a novel protein kinase C inhibitor.** *J Biol Chem* 1993, 268:1982–1986.

8. Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B, Barsoum J: **Tat-mediated delivery of heterologous proteins into cells.** *Proc Natl Acad Sci USA* 1994, 91:664–668.

9. •• Liu X-Y, Timmons S, Lin Y-Z, Hawiger J: **Identification of a functionally important sequence in the cytoplasmic tail of integrin b3 by using cell-permeable peptide analogs.** *Proc Natl Acad Sci USA* 1996, 93:11819–11824.

This paper provides an example of the power of cell-permeable peptide import to conduct structure-function analysis of intracellular segment of adhesion receptors, integrins β_3, and β_1 in human erythroleukemia cells, endothelial cells and fibroblasts.

10. • Lin YZ, Yao SY, Hawiger J: **Role of the nuclear localization sequence in fibroblast growth factor-1-stimulated mitogenic pathways.** *J Biol Chem* 1996, 271:5305–5308.

This paper, along with [19•], shows how the action of two distinct growth factors FGF and EGF can be analyzed using noninvasive cell-permeable peptide import.

11. Hoyt DW, Gierasch LM: **Hydrophobic content and lipid interactions of wild-type and mutant OmpA signal peptides correlate with their *In vivo* function.** *Biochemistry* 1991, 30:10155–10163.

12. Andrews DW, Young JC, Mirels LF, Czarnota GJ: **The role of the N region in signal sequence and signal-anchor function.** *J Biol Chem* 1992, 267:7761–7769.

13. Fra AM, Williamson E, Simons K, Parton RG: **Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae.** *J Biol Chem* 1994, 269:30745–30748.

14. Clark EA, Brugge JS: **Integrins and signal transduction pathways: the road taken.** *Science* 1995, 268:233–239.

15. Dingwall C, Laskey RA: **Nuclear targeting sequences – a consensus? [see comments].** *Trends Biochem Sci* 1991, 16:478–481.

16. Boulikas T: **Putative nuclear localization signals (NLS) in protein transcription factors.** *J Cell Biol* 1994, 55:32–58.

17. Powers MA, Forbes DJ: **Cytosolic factors in nuclear transport: what's importin?** *Cell* 1994, 79:931–934.

18. Imamura T, Engleka K, Zhan X, Tokita Y, Forough R, Roeder D, Jackson A, Maier JA, Hla T, Maciag T: **Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence.** *Science* 1990, 249:1567–1570.

19. • Rojas M, Yao SY, Lin YZ: **Controlling epidermal growth factor (EGF)-stimulated Ras activation in intact cells by a cell-permeable peptide mimicking phosphorylated EGF receptor.** *J Biol Chem* 1996, 271:27456–27461.

See annotation [10•].

20. Joliot A, Pernelle C, Deagostini-Bazin H, Prochiantz A: **Antennapedia homeobox peptide regulates neural morphogenesis.** *Proc Natl Acad Sci USA* 1991, 88:1864–1868.

21. Joliot AH, Triller A, Volovitch M, Pernelle C, Prochiantz A: **Alpha-2,8-polysialic acid is the neuronal surface receptor of antennapedia homeobox peptide.** *New Biol* 1991, 3:1121–1134.

22. Theodore L, Derossi D, Chassaing G, Llirbat B, Kubes M, Jordan P, Chneiweiss H, Godement P, Prochiantz A: **Intraneuronal delivery of protein kinase C pseudosubstrate leads to growth cone collapse.** *J Neurosci* 1995, 15:7158–7167.

23. Troy CM, Derossi D, Prochiantz A, Greene LA, Shelanski ML: **Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide-peroxynitrite pathway.** *J Neurosci* 1996, 16:253–261.

24. Troy CM, Stefanis L, Prochiantz A, Greene LA, Shelanski ML: **The contrasting roles of ICE family proteases and interleukin-1 beta in apoptosis induced by trophic factor withdrawal and by copper/zinc superoxide dismutase down-regulation.** *Proc Natl Acad Sci USA* 1996, 93:5635–5640.

25. Sheldon K, Liu D, Ferguson J, Gariepy J: **Loligomers: design of *de novo* peptide-based intracellular vehicles.** *Proc Natl Acad Sci USA* 1995, 92:2056–2060.

26. Kolson DL, Buchhalter J, Collman R, Hellmig B, Farrell CF, Debouck C, Gonzalez-Scarano F: **HIV-1 Tat alters normal organization of neurons and astrocytes in primary rodent brain cell cultures: RGD sequence dependence.** *AIDS Res Hum Retroviruses* 1993, 9:677–685.

27. Lafrenie RM, Wahl LM, Epstein JS, Hewlett IK, Yamada KM, Dhawan S: **HIV-1-Tat modulates the function of monocytes and alters their interactions with microvessel endothelial cells. A mechanism of HIV pathogenesis.** *J Immunol* 1996, 156:1638–1645.

28. Albini A, Barillari G, Benelli R, Gallo RC, Ensoli B: **Angiogenic properties of human immunodeficiency virus type 1 Tat protein.** *Proc Natl Acad Sci USA* 1995, 92:4838–4842.

29. Li CJ, Friedman DJ, Wang C, Metelev V, Pardee AB: **Induction of apoptosis in uninfected lymphocytes by HIV-1 Tat protein.** *Science* 1995, 268:429–431.

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Cell Internalization of the Third Helix of the Antennapedia Homeodomain Is Receptor-independent*

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We have recently reported that a 16-amino acid long polypeptide corresponding to the third helix of the DNA binding domain (homeodomain) of Antennapedia, a *Drosophila* transcription factor, is internalized by cells in culture (Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) *J. Biol. Chem.* 269, 10444–10450). The capture of the homeodomain and of its third helix at temperatures below 10 °C raised the problem of the mechanism of internalization. The present demonstration, that a reverse helix and a helix composed of D-enantiomers still translocate across biological membranes at 4 and 37 °C strongly suggests that the third helix of the homeodomain is internalized by a receptor-independent mechanism. The finding that introducing 1 or 3 prolines in the structure does not hamper internalization also demonstrates that the α-helical structure is not necessary. The data presented are compatible with a translocation process based on the establishment of direct interactions with the membrane phospholipids. The third helix of the homeodomain has been used successfully to address biologically active substances to the cytoplasm and nucleus of cells in culture (Théodore, L., Derossi, D., Chassaing, G., Llirbat, B., Kubes, M., Jordan, P., Chneiweiss, H., Godement, P., and Prochiantz, A. (1995) *J. Neurosci.* 15, 7158–7167). Therefore, in addition to their physiological implications (Prochiantz, A., and Théodore, L. (1995) *BioEssays* 17, 39–45), the present results open the way to the molecular design of cellular vectors.

Homeoproteins are transcription factors involved in several important biological processes occurring primarily, but not exclusively, during development. The DNA binding domain of these transcription factors is highly conserved and is called the homeodomain. It consists of 60 amino acids arranged in 3 α-helices. Helix 3 is separated from helix 2 by a β turn and is called the recognition helix, because it is involved in the interaction of the homeodomain with specific sites in the large groove of double-stranded DNA (Gehring *et al.*, 1994).

In the course of our studies on the role of homeoproteins in neuronal development we observed that the homeodomain of Antennapedia, a *Drosophila* homeoprotein, is internalized by cells in culture and, following internalization, is conveyed to cell nuclei where it can directly and specifically interfere with transcription (Bloch-Gallego *et al.*, 1993; Joliot *et al.*, 1991a, 1991b; Le Roux *et al.*, 1993, 1995). Internalization occurs at both 4 and 37 °C and thus does not seem to involve classical receptor-mediated endocytosis.

The region of the homeodomain (AntpHD)[1] of the *Drosophila* transcription factor responsible for its internalization by cells in culture, in particular nerve cells, has been mapped to its third helix (Le Roux *et al.*, 1993). This finding has led to the demonstration that a 16-amino acid long peptide corresponding to the third helix of the homeodomain (minus an N-terminal glutamate residue) translocates at 37 and 4 °C across biological membranes, reaches the cytoplasm, and is eventually conveyed to the nucleus of cells in culture (Derossi *et al.*, 1994).

The successful use of this peptide as a vector to address biologically active compounds inside live cells (Théodore *et al.*, 1995; Troy *et al.*, 1996) was a strong incentive to start experiments aimed at understanding its mechanism of translocation. Our previous studies (Derossi *et al.*, 1994) had led to the conclusion that the peptide is poorly structured in water but adopts an amphipathic α-helical structure in a hydrophobic environment. We also observed a tendency of the peptide to form dimers and even multimers in the presence of SDS, a phenomenon tentatively attributed to the formation of and association with detergent micelles. In the present study we report new data demonstrating that internalization does not require specific interactions with a chiral receptor (binding site or transporter) or the formation of a charged pore by an α-helical conformation of the peptide.

MATERIALS AND METHODS

Peptides Synthesis—Peptide synthesis was carried out on a 0.1 mmol scale (ABI Model 431A synthesizer) starting from an α-*p*-methylbenzhydrylamine resin (typical substitution, 0.77). All *N*-α-*t*-butoxycarbonyl amino acids, in 10-fold excess, were assembled using dicyclohexylcarbodiimide and 1-hydroxybenzotiazol as coupling agents. After removal of the last *N*-*t*-butoxycarbonyl protecting group of 5-aminopentanoic acid, the *N*-hydroxysuccinimido ester of biotin was coupled manually in dimethylformamide. Peptides were cleaved from the resin by anhydrous fluorhydic acid and purified by preparative reverse phase HPLC (Applied Biosystems), using a 10 × 250-nm Bronwlee column (Aquapore Octyl, 300 Å pore size) using various acetonitrile gradients in aqueous 0.1% trifluoroacetic acid. The purity of collected fractions was established by analytical isocratic separation (HPLC, Waters Associate) on lichrosphere 10 RP-8 columns (Merck) and in 0.25 M triethylammonium phosphate, pH 3.0 (buffer A), and acetonitrile. Peptide molecular weights were determined by electrospray ionization mass spectrometry in the laboratory of Pr. J. C. Tabet (Université P. et M. Curie, Paris). All peptides are presented in Fig. 1 and as follows: AntpHD(43-58) abbreviated 43-58; purification (gradient: 12–22, 8% CH_3CN, linear, 30 min); purity >95%; HPLC (iso 19.2% CH_3CN in buffer A), 17.3 min. Ant-

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[1] The abbreviations used are: AntpHD, Antennapedia homeodomain; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; iso, isocratic.

EXHIBIT 3

pHD(58-43) abbreviated 58-43; purification (gradient: 12–22, 8% CH_3CN, linear, 30 min); purity >95%; HPLC (iso 19.8% CH_3CN in buffer A), 11 min. D-AntpHD(43-58) abbreviated D43-58; purification (gradient: 12–22, 8% CH_3CN, linear, 30 min); purity >95%; HPLC (iso 21% CH_3CN in buffer A), 9 min. (Pro50)AntpHD(43-58) abbreviated Pro50; purification (gradient: 12–22, 8% CH_3CN, linear, 30 min); purity >95%; HPLC (iso 20, 4% CH_3CN in buffer A), 12 min. (Pro-45, Pro-50, Lys-54, Pro-55)AntpHD(43-58) abbreviated 3Pro, purification (gradient: 12–22, 8% CH_3CN, linear, 30 min); purity >95%; HPLC (iso 15, 6% CH_3CN in buffer A), 10 min. Peptide AntpHD(41-55) abbreviated 41-55 was described in Derossi *et al.*, (1994).

Cell Cultures—The cell culture conditions have been described in earlier reports (Lafont *et al.*, 1992). Briefly, small fragments from the cortical-striatal region of the embryonic rat brain between E13 and E15 were incubated in trypsin (0.25%; Life Technologies, Inc.) for 5 min at 37 °C, washed twice in phosphate buffer containing 33 mM glucose (PBS), and incubated for 10 min at 37 °C with 15 μg/ml DNase I (Sigma) in Dulbecco's modified Eagle's medium/F12 supplemented with 33 mM glucose, 2 mM glutamine, 10 mM Hepes, pH 7.4, 3 mM $NaHCO_3$, penicillin 5 units/ml, and streptomycin 5 μg/ml (SFM). The cells were dissociated mechanically, washed three times in SFM, and plated at a concentration of 25,000 cells/cm² on glass coverslips (16-mm diameter) coated with 15 μg/ml DL-polyornithine (Sigma). All cultures were in chemically defined medium consisting of SFM supplemented with 0.1% ovalbumin, 100 μg/ml transferrin, 20 nM progesterone, 20 μM putrescine, and 30 nM selenium.

AntpHD(43-58) = **43-58**

Biot-Apa-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2
43 58

AntpHD(41-55) = **41-55**

Biot-Apa-Thr-Glu-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-NH_2
41 55

AntpHD(58-43) = **58-43**

Biot-Apa-Lys-Lys-Trp-Lys-Met-Arg-Arg-Asn-Gln-Phe-Trp-Ile-Lys-Ile-Gln-Arg-NH_2
43 58

D-AntpHD(43-58) = **D43-58**

Biot-Apa-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2
43 58

[Pro^{50}]AntpHD(43-58) = **Pro50**

Biot-Apa-Arg-Gln-Ile-Lys-Ile-Trp-Phe-Pro-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys-NH_2
43 58

[Pro^{45},Pro^{50},Lys^{54},Pro^{55}]AntpHD(43-58) = **3Pro**

Biot-Apa-Arg-Gln-Pro-Lys-Ile-Trp-Phe-Pro-Asn-Arg-Arg-Lys-Pro-Trp-Lys-Lys-NH_2
43 58

FIG. 1. **Primary sequences of the different polypeptides used in this study.** *Apa*, aminopentanoic acid; *Biot*, biotin.

Peptide Internalization and Visualization—When added to cells in culture, peptides were diluted in the chemically defined medium supplemented with 10% fetal calf serum and the following mixture of protease inhibitors: 0.5 mM Pefablock, 1 μg/ml α_2-macroglobulin, and 10 μg/ml leupeptin. 2–4 h after the addition of a volume of peptide equal to the volume present in the dishes, the cells were washed three times with the chemically defined medium, fixed for 5 min in 4% paraformaldehyde at room temperature, and then for 5 min at −20 °C in ethanol/CH_3COOH (95/5), washed three times in PBS, and incubated for 30 min with fluorescein-linked streptavidin (Amersham Corp.) diluted 1000-fold into PBS plus 10% newborn calf serum (Life Technologies, Inc.). At the end of the incubation the cells were washed three times in PBS, once in water, dried, and mounted in Mowiol for examination.

In some experiments freshly dissociated cells were resuspended in 0.6 ml of PBS or chemically defined medium containing appropriate peptide concentrations plus protease inhibitors. After 2–4 h of incubation with regular gentle shaking, the cells were centrifuged and washed once with 1 ml of PBS plus protease inhibitors and a second time with PBS adjusted to 0.5 M NaCl. The final cellular pellet was directly resuspended in Laemmli buffer or sonicated to allow for a brief purification of the peptides on streptavidin-agarose (Life Technologies, Inc.). The cells or the beads were boiled and frozen for storage at −80 °C or immediately loaded onto a 12 to 22% polyacrylamide SDS gradient gel. Following migration, peptides were electrotransferred onto Immobilon (Amersham Corp.) in 25 mM Tris, 192 mM glycine, pH 8.8, the filter was fixed in 4% glutaraldehyde for 15 min, and nonspecific binding sites were blocked by a 2-h incubation in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.2% Tween 20, and 4% bovine serum albumin. The blots were incubated 1 h at room temperature with streptavidin-peroxidase (Amersham Corp.) diluted 1 to 1000 in the blocking solution with 0.5% bovine serum albumin, washed several times with the same solution, and revealed with luminol, in accordance with the instructions of the manufacturer (Amersham Corp.).

Confocal Microscopy—Data were obtained with a confocal laser scanning microscope Sarastro 2000 (Molecular Dynamics). Excitation was with an argon ion laser set at 488 nm for fluorescein isothiocyanate excitation, and the emitted light was filtered with an appropriate long pass filter (510 nm). Sections presented were taken approximately at the mid-height level of the cells. Photomultiplier gain and laser power were identical within each experiment.

ELISA Quantification—The cells were incubated with the different peptides as described previously. After several washes with PBS, approximately 5 × 10^4 cells per condition were plated on ELISA wells. Cells were left to attach for 2 h, fixed overnight in 5% paraformaldehyde at 4 °C, permeabilized in methanol, and rinsed three times in PBS. Endogenous alkaline phosphatase was neutralized by incubating the

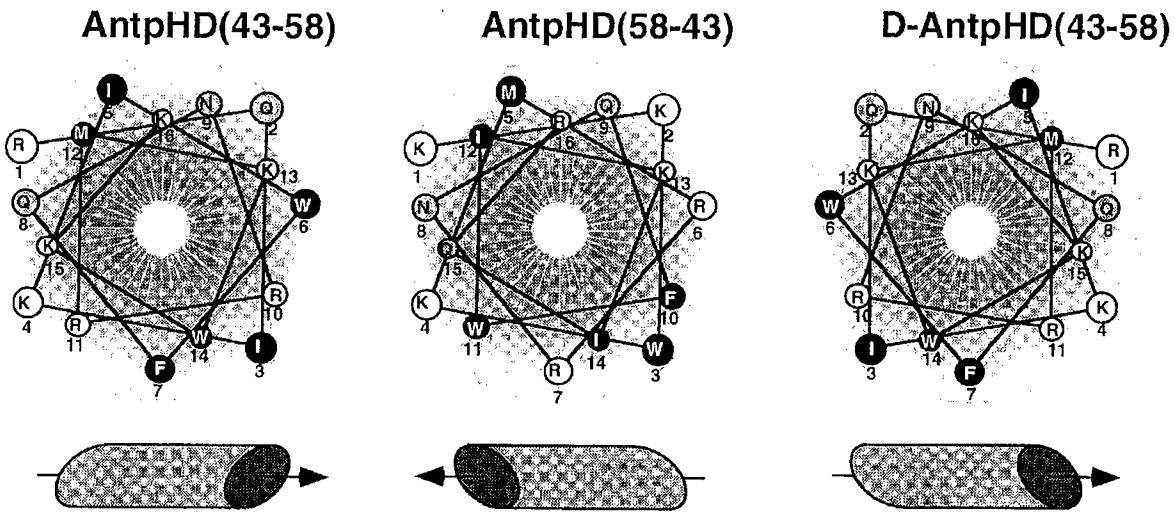

FIG. 2. **Schematic structural representation of peptides 43-58, 58-43 and D43-58.** *Above*, helical plot of the peptides; *below*, schematic representation of the three peptides in α-helical conformation. *Arrowhead*, C terminus.

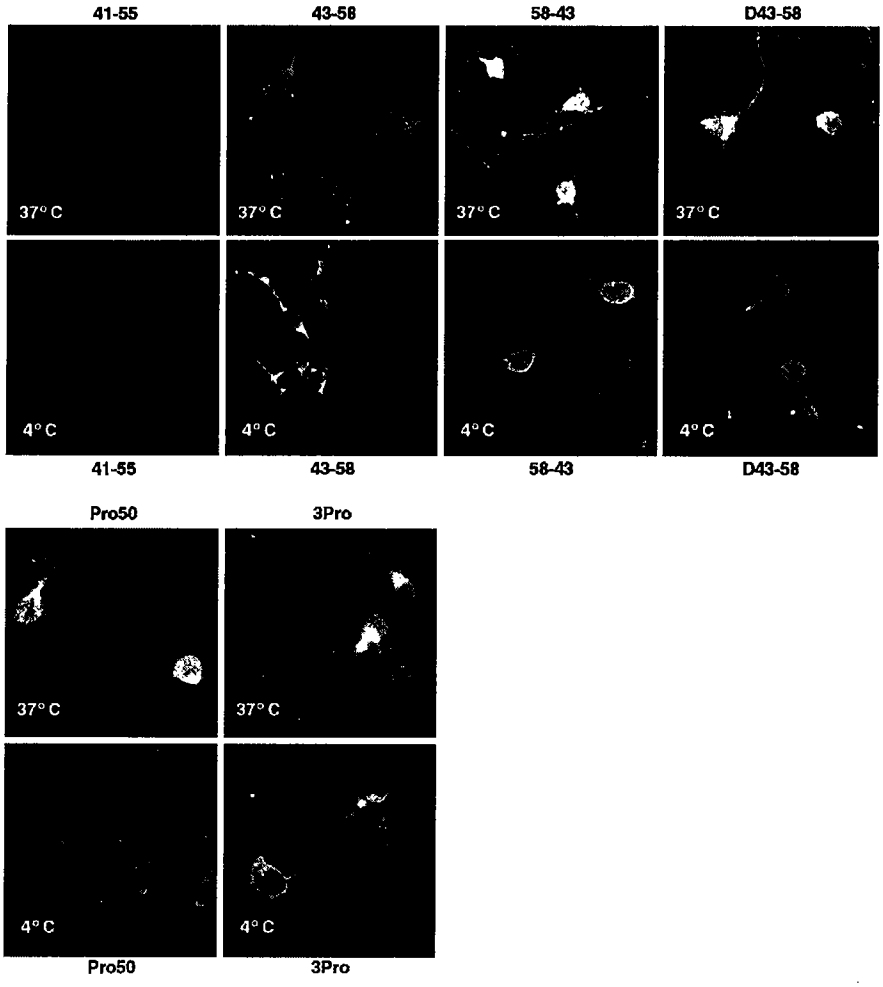

FIG. 3. **Peptide visualization with confocal microscopy.** Cortical-striatal cells dissociated from rat E15 cells were cultured for 2 days at a density of 25,000 cells/cm^2 before addition of the different peptides at a concentration of 22 μM. After 2 h of incubation at 37 or 4 °C, the cells were rinsed and fixed, and the presence of biotin was revealed with fluorescent streptavidin. Sections presented here demonstrate that all peptides except peptide 41-55 are internalized at both temperatures. Note that at 4 °C proline-containing peptides (Pro50 and 3Pro) are not found in the nuclei. Laser power is identical within each experiment, photomultiplier gain is reduced 4-fold for peptide 58-43 at both temperatures.

ELISA plate at 65 °C for 1 h. After blocking in 100 mM Tris-HCl, pH 7.4, NaCl 0.3 M, 0.2% Tween, and 5% milk, cells were incubated for 30 min, at room temperature, with alkaline phosphatase-streptavidin (Vectastain ABC kit). Alkaline phosphatase activity was measured spectrophotometrically at 405 nm using a freshly made solution of 1 mg/ml nitrophenyl phosphate (Sigma) in 10 mM diethanolamine (pH 9.5), 0.5 mM $MgCl_2$.

Electron Microscopy—The cells (2×10^6/ml) were incubated for 2 h at 37 °C with the peptides. After several washes with culture medium, the cellular pellets were included in a collagen gel (50% collagen in culture medium), fixed for 30 min at 4 °C in PBS containing 4% paraformaldehyde and 0.1% glutaraldehyde, washed in PBS, incubated for 30 min at 4 °C in PBS containing 0.3% NH_4Cl, and rinsed overnight at 4 °C with PBS. Progressive lowering of temperature was performed in an AFS Reichert (Leica) apparatus. This procedure involves stepwise reductions in temperature (20 to −35 °C) as the concentration of the dehydrating agents is increased (30 to 95% ethanol). The infiltration in resin (Lowicryl K4M, Boiziau distribution) was performed at −35 °C by progressively increasing the resin concentration and decreasing the percentage of absolute ethanol. Polymerization was carried out at −35 °C for 48 h using reflected light from an ultraviolet source. The temperature was slowly increased to reach 20 °C. Ultrathin sections from the different blocks were prepared and mounted on 400 mesh Formvar-coated nickel grids. Immunodetection was performed as follows: pretreatment in PBS containing 5% bovine serum albumin for 30 min, washes in 0.1% bovine serum albumin-PBS, incubation for 1 h in PBS containing 0.01% gelatin, 1/200 colloidal gold streptavidin conjugate (15 nm, British Bio Cell International), washes in PBS and H_2O, fixation for 5 min in 1% glutaraldehyde, and washes in H_2O. After air drying, sections were contrasted using 4% uranyl acetate in water and Millonig's lead acetate-tartrate. The samples were analyzed with a Philips 400 electron microscope.

RESULTS

Peptide Internalization Is Not Receptor-mediated—To investigate whether 43-58 internalization requires a chiral receptor, we synthesized peptides 43-58D and 58-43. Peptide 43-58D has a primary sequence identical to that of 43-58 but is entirely composed of D-enantiomers. Peptide 58-43 is composed of the same amino acids as peptide 43-58, but the order of the amino acids has been reversed, thus leading to a totally different primary structure. Fig. 2, in which the peptides are represented in a α-helical conformation, illustrates that the modifications of the original peptide modify the orientation of the helix but not its amphipathic profile.

Peptide internalization was tested on nerve cells after 2 days of development *in vitro*. Peptides at a concentration of 22 μM were added for 2 h at 4 °C or 37 °C. Peptide 41-55, which is not internalized (Derossi *et al.*, 1994), was used as a control. After several washes in PBS, including a final wash in PBS adjusted to 0.5 M NaCl, the cells were fixed and the presence of biotin revealed with fluorescein isothiocyanate-labeled streptavidin. Fig. 3 (*top panel*) illustrates that the 3 peptides, in contrast to

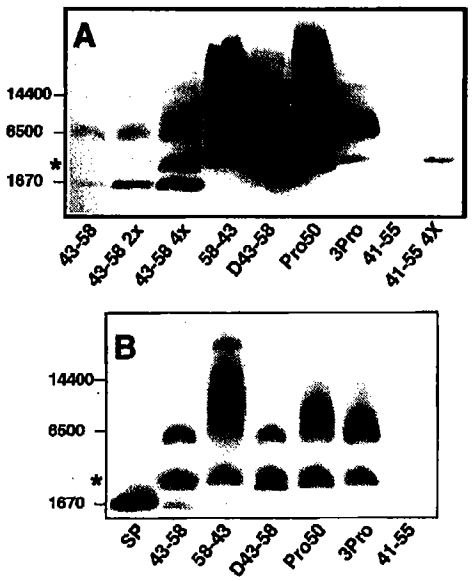

FIG. 4. **Gel electrophoresis of cell extracts.** E15 cortical-striatal cells at (1.2×10^6 cells in 0.6 ml) were incubated with the different peptides for 2 h at 37 °C (*A*) or 4 °C (*B*). After several washes, the intracellular presence of the peptides was analyzed by gel electrophoresis and electrotransfer. Note that all peptides have a tendency to form multimers. Multimerization is in part due to the formation of micelles in SDS (Derossi *et al.*, 1994). The degree of multimerization is not similar with all peptides probably because of differences between peptide-peptide interactions. For example, it was observed by NMR (G. Chassaing, unpublished results) that a prolin in 50 provokes the construction of the N-terminal domain of the peptide into a β-sheet, thus favoring peptide dimerization. *SP*, substance P. Because of their spontaneous degradation at 37 °C (as seen on the gel) twice (*2×*) or four times (*4×*) the amount of 43-58 or of 41-55 were added to the cells (in *A*).

41-55, are internalized by the cells at both temperatures.

To establish that the internalized peptides were not degraded, the peptides retrieved by incubating the cellular extracts on streptavidin-agarose were separated by SDS-PAGE and blotted, and their presence was revealed with streptavidin-peroxidase (see "Materials and Methods" for details). Fig. 4 demonstrates that 58-43 and 43-58D are internalized at 37 °C (Fig. 4*A*) and 4 °C (Fig. 4*B*). All peptides run as monomers (*asterisks*), dimers, and multimers on an SDS gel. For size comparison, the 11-amino acid long substance P peptide (molecular mass, 1670 Da), aprotinin (molecular mass, 6500 Da), and lysozyme (molecular mass, 14400 Da) were run in parallel. Note that peptide 41-55 is not internalized and that peptide 43-58 is highly sensitive to degradation at 37 °C but not at 4 °C.

This first series of experiments strongly suggested that peptide internalization is not receptor-dependent. To better establish this point, we compared the localization of 43-58 and 43-58D at the ultrastructural level. Fig. 5 illustrates that the two peptides can be localized within the cells, both in the cytoplasm and in the nucleus. The presence of gold particles in endocytic figures was rarely observed.

Is the α-Helical Structure Important?—To test the importance of the helical structure for internalization, two peptides were synthesized into which were introduced one or three prolines. In Pro50, the glutamine in position 50 was replaced by a proline. In 3Pro, in addition to Gln-50, Ile-45, and Lys-55 were also replaced by proline residues. As illustrated in Fig. 3, introducing one or three prolines into the sequence did not hamper internalization at 4 or 37 °C, suggesting that the positions modified are not important and that a helical structure is not mandatory for translocation.

That the peptides were intact after internalization was verified by gel electrophoresis and Western blotting of the peptides retrieved from the cells and purified on streptavidin-agarose. As demonstrated in Fig. 4, the two peptides migrate on the gels with electrophoretic mobilities which indicate that they are intact and suggest that they can form dimeric and multimeric structures.

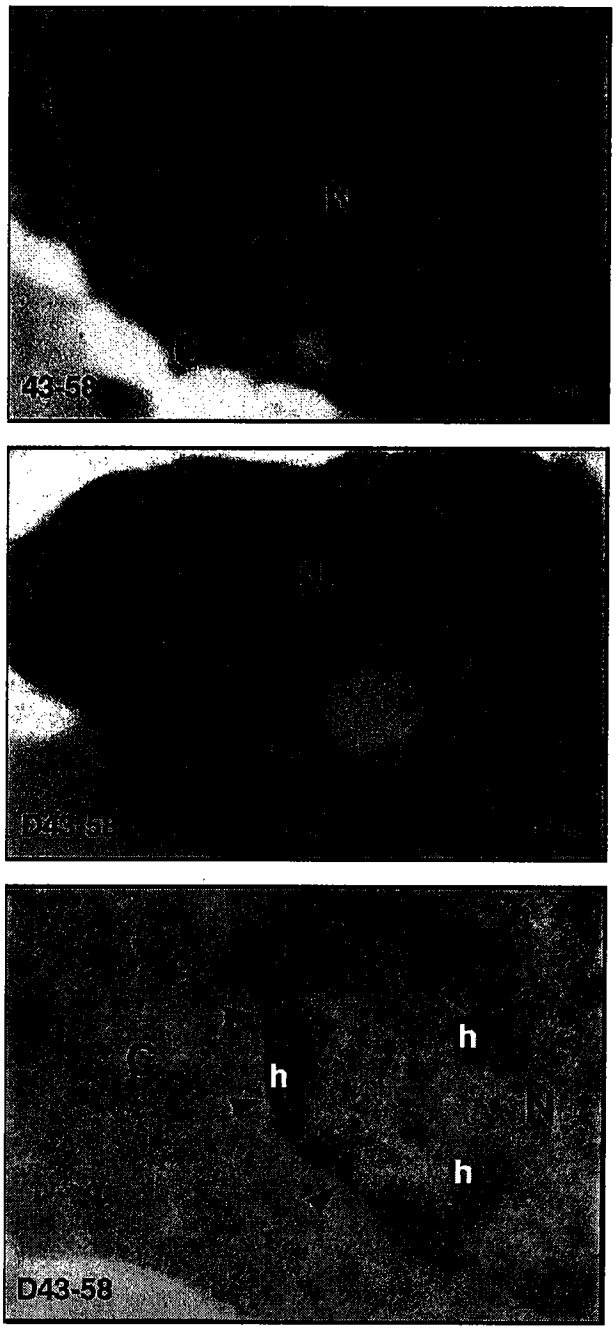

FIG. 5. **Peptides visualization by electron microscopy.** Cortical-striatal cells (2×10^6 cells/ml) dissociated from E15 rat embryos were incubated with 44 μM of peptides 43-58 or D43-58 for 2 h at 37 °C. After incubation, cells were washed, inoculated in collagen gel, and analyzed by electron microscopy (see "Materials and Methods" for details). Note that both peptides accumulate primarily in the cytoplasm (*C*) and nucleus (*N*) and, within the nucleus, in the heterochromatin (*h*). *Arrowheads* underline the nuclear membrane.

Although the translocation at 4 and 37 °C allows for the addressing of the two peptides to the cytoplasmic compart-

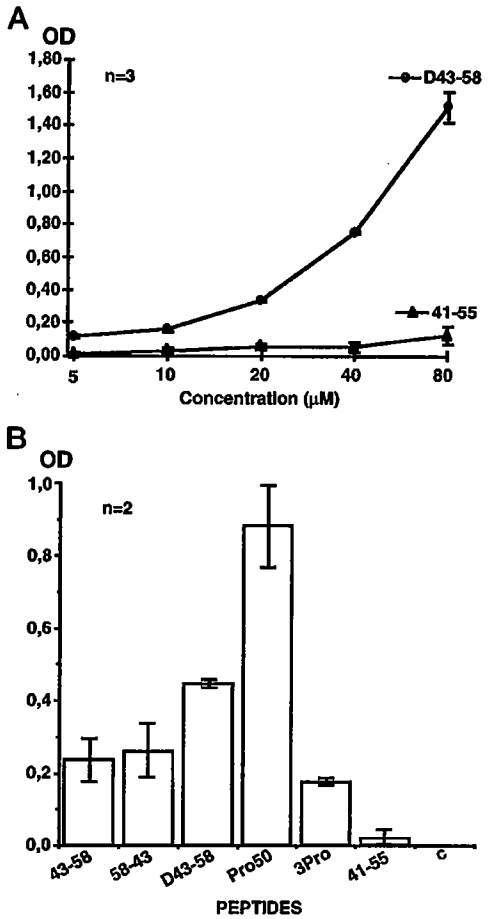

FIG. 6. **ELISA quantification of internalized peptides.** *A*, increasing concentrations of peptides D43-58 and 41-55 were incubated in suspension for 2 h with 10^6 cortical-striatal neurons in a final volume of 1 ml. At the end of the incubation period, the cells were rinsed and plated in ELISA wells. After fixation, the presence of biotin was detected with alkaline phosphatase-streptavidin (n = 3). Two independent experiments gave similar results. *Bar* = standard error of means. *B*, 10^6 cortical-striatal neurons were incubated with the different peptides at 44 μM for 2 h at 4 °C. At the end of the incubation period, the cells were rinsed and plated in ELISA wells. After fixation, biotin was detected with alkaline phosphatase-streptavidin (n = 2). The mean and the two values (*horizontal bars*) are indicated. Two independent experiments gave similar results

ment, we noted that peptides Pro50 and 3Pro were not conveyed to the nuclei to the same extent as were the other peptides. This latter point is illustrated in Fig. 3. It is shown that, compared to 58-43, which is present throughout the cytoplasm and nuclei of the cells, 3Pro is concentrated in the cytoplasm. Pro50 behaved similarly to 3Pro, whereas the distributions of 43-58 or 43-58D are identical to that of 58-43.

Quantification—The patterns of staining observed after internalization as well as the profiles of the Western blots suggested that all peptides were not internalized with the same efficiency. However, the small size of the peptides and the number of steps necessary to obtain a Western blot precluded the use of the latter approach to compare rigorously the internalization of the different peptides. To estimate better the amount of peptide internalized, an ELISA test was developed (see "Material and Methods" for details). In a preliminary experiment we used the 43-58D, which, because of the D-enantiomers is unlikely to be rapidly degraded, to define an optimal concentration to be used for this analysis. As shown in Fig. 6*A*, 43-58D internalization (2 h at 37 °C) increases linearly with

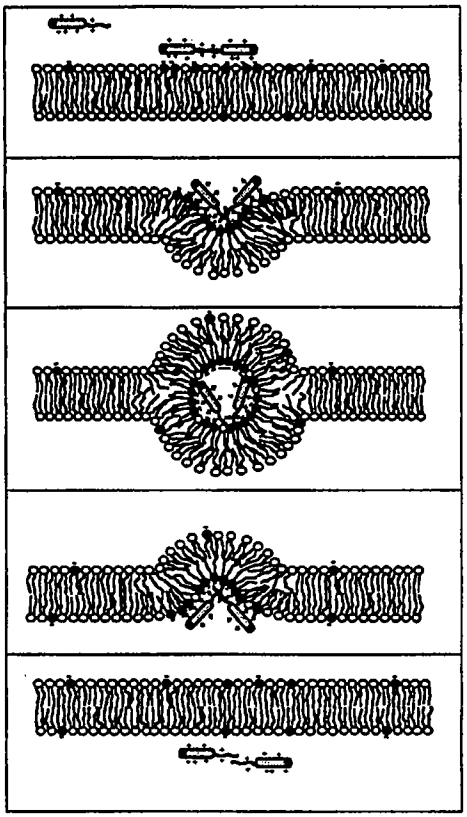

FIG. 7. **Model of internalization.** In this model the peptide, arbitrarily represented as a dimer, recruits negatively charged phospholipids (*filled circles*) and induces the formation of an inverted micelle. The hydrophilic cavity of the micelle accommodates the peptide and, possibly, sequences attached to it that can subsequently be released in the cytoplasmic compartment.

concentration and does not saturate between 10 and 80 μM.

The comparison between the different peptides used in this study was thus done at a concentration of 40 μM and both at 4 and 37 °C. Similar results were obtained at the two temperatures except for 43-58 which, as already mentioned, is subject to proteolytic degradation at the highest temperature. Fig. 6*B* illustrates the results of such an experiment, done at 4 °C. It confirms that 41-55 is not internalized. In addition, it suggests that all peptides are internalized with a similar efficiency, except for Pro50, for which the amount recovered inside the cells is significantly and reproducibly higher.

DISCUSSION

The strongest evidence against receptor-mediated endocytosis is the internalization of 43-58D and 58-43. The two peptides are very unlikely to interact specifically with a receptor that would recognize a precise sequence of amino acids (Wade *et al.*, 1990). We cannot, however, entirely exclude the possibility that a receptor or a family of receptors would recognize a general structure conserved between the three polypeptides, in particular a defined organization of hydrophobic and charged amino acids. Even if such interactions exist, the formation of endocytotic coated vesicles is not compatible with internalization at 4 °C. In addition, endocytotic figures were almost never observed at the ultrastructural level.

NMR studies demonstrated (not shown) that in Pro50 the 43-50 domain is in a β-sheet conformation, the α-helix being maintained from residue 51 to residue 58. Although we do not have the corresponding NMR data, it is highly unlikely that the presence of three prolines would be compatible with an α-hel-

ical structure. We thus propose that the α-helical structure observed in a hydrophobic environment (Derossi *et al.*, 1994) is not necessary for the internalization of the peptides.

Peptides with prolines reach the cytoplasm but are poorly conveyed to the nucleus. This suggests that nuclear accumulation is not only due to the small size of the peptide but also to its sequence and/or structure. The notion that the highly basic third helix is part of a nuclear localization signal for homeoproteins is supported by homologies with the *Mat-α2* transcription factor the nuclear localization signal of which is in the homeodomain (Hall *et al.*, 1990) and by results showing that deleting its homeodomain blocks the nuclear import of Hoxa-5.[2] In addition to its possible physiological significance regarding the structure of the nuclear localization signal, the primarily cytoplasmic accumulation of Pro50 and 3Pro might be useful for the development of vectors that would preferentially address drugs in the cytoplasm.

That translocation is not due to an α-helical amphipathic conformation leading to the formation of a positively charged channel is further supported by the following facts. First, a 16-amino acid α-helix is not long enough to span the plasma membrane; second, we could never measure the passage of a current that would suggest the formation of a channel[3]; third, replacing the two tryptophan residues by two phenylalanines does not modify the helical structure or the amphipathicity, but virtually abolishes translocation (Derossi *et al.*, 1994). Finally, translocation of long polypeptides or oligonucleotides attached to the helix through a positively charged pore (Allinquant *et al.*, 1995; Chatelin *et al.*, 1995; Perez *et al.*, 1994; Troy *et al.*, 1996a 1996b; Fåhraeus *et al.*, 1996) is very unlikely.

The two main possibilities that remain to explain peptide translocation are the formation of inverted micelles and fluid phase pinocytosis (de Kruijff *et al.*, 1985; Illinger *et al.*, 1991; Sandvig and van Deurs, 1994). Indeed, both phenomena are receptor-independent and can occur at 4 °C. The reasons for favoring a model implying inverted micelles (Fig. 7) are as follows. First, endocytosis figures are rarely seen at the ultrastructural level; second, as opposed to results obtained with the glycophosphatidylinositol linked folate receptor (Leamon and Low, 1991), the size of the polypeptides that can be internalized after attachment to the third helix is limited and, for a similar molecular weight, depends on the structure of the peptide attached to the homeodomain or to its third helix (Chatelin *et al.*, 1996; Perez *et al.*, 1994); third, tryptophans, which are necessary for translocation (Derossi *et al.*, 1994), are known inducers of inverted micelles (de Kruijff *et al.*, 1985); fourth, the formation of peptide multimers in the presence of SDS could reflect micelle formation (Derossi *et al.*, 1994). Finally, phosphorus NMR studies in which we studied the interactions of 43-58 (which is internalized) and of 43-58 in which tryptophans are replaced by phenylalanines (poorly internalized) (Derossi *et al.*, 1994) with brain phospholipids demonstrated that only the former peptide provokes the formation of hexagonal phases or inverted micelles.[4]

The interest of the present study is 2-fold. First, it is potentially important to the development of new vector systems for the intracellular addressing of pharmacological substances. The discovery that the homeodomain of Antennapedia, and within this structure its third helix (peptide 43-58), could be used to transport polypeptides and oligonucleotides into cells has now permitted the identification of structural features responsible for this unexpected property. Although we do not yet understand the details of the mechanism of translocation, the simple fact of being able to fabricate a D-helix unlikely to be rapidly degraded, or variants (Pro50 and 3Pro) that could address exogenous molecules exclusively to the cytoplasm is of obvious interest for the study of intracellular functions.

A second point of interest is the physiological significance of homeodomain and homeoprotein internalization. Although the internalization and even the nuclear addressing of exogenous proteins has been noted before (Prochiantz and Théodore, 1995; Rubartelli *et al.*, 1990), it is seldom that a systematic study of the structure and mechanisms involved in these processes has been undertaken. It is striking that, in the case of Antennapedia, the structure which is necessary for translocation corresponds to the third helix of the homeodomain. Because this helix is highly conserved among several homeodomains, it is possible that membrane translocation is a property shared by several homeodomains. This latter point is supported by experiments showing that the homeodomains of Engrailed, fushitarazu, and Hoxa-5 are internalized (Chatelin *et al.*, 1996). More interesting is the finding that full-length Hoxa-5 (Chatelin *et al.*, 1996) and Hoxc-8[3] are also internalized and targeted to the nucleus of cells in culture. Our proposal is that the third helix of a homeodomain could induce the formation of an inverted micelle and thus of an hydrophilic cavity capable to accommodate an entire homeoprotein. It has to be placed in the perspective of the hypothesis that cells may exchange positional information through the local trading of homeoproteins (Chatelin *et al.*, 1995; Prochiantz and Théodore, 1995).

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REFERENCES

Allinquant, B., Hantraye, P., Mailleux, P., Moya, K., Bouillot, C., and Prochiantz, A. (1995) *J. Cell Biol.* **128**, 919–927
Bloch-Gallego, E., Le Roux, I., Joliot, A. H., Volovitch, M., Henderson, C. E., and Prochiantz, A. (1993) *J. Cell Biol.* **120**, 485–492
Chatelin, L., Volovitch, M., Joliot, A. H., Perez, F., and Prochiantz, A. (1996) *Mech. Dev.* **55**, 111–117
de Kruijff, B., Cullis, P. R., Verkleij, A. J., Hope, M. J., van Echteld, C. J. A., Taraschi, T. F., van Hoogevest, P., Killian, J. A., Rietvel, A., and van der Steen, A. T. M. (1985) *Progress in Protein-Lipid Interactions*, pp. 89–142, Elsevier Science Publishers B.V., Amsterdam
Derossi, D., Joliot, A. H., Chassaing, G., and Prochiantz, A. (1994) *J. Biol. Chem.* **269**, 10444–10450
Fåhraeus, R., Paramio, J. M., Ball, K. L., Lain, S., and Lane, D. P. (1996) *Curr. Biol.* **6**, 84–91
Gehring, W. J., Affolter, M., and Gürglin, T. (1994) *Annu. Rev. Biochem.* **63**, 487–526
Hall, M. N., Craik, C., and Hiraoka, Y. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 6954–6958
Illinger, D., Poindron, P., and Kuhry, J. G. (1991) *Biol. Cell* **73**, 131–138
Joliot, A., Pernelle, C., Deagostini-Bazin, H., and Prochiantz, A. (1991a) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 1864–1868
Joliot, A., Triller, A., Volovitch, M., Pernelle, C., and Prochiantz, A. (1991b) *New Biol.* **3**, 1121–1134
Lafont, F., Rouget, M., Triller, A., Prochiantz, A., and Rousselet, A. (1992) *Development* **114**, 17–29
Le Roux, I., Joliot, A. H., Bloch-Gallego, E., Prochiantz, A., and Volovitch, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 9120–9124
Le Roux, I., Duharcourt, S., Volovitch, M., Prochiantz, A., and Ronchi, E. (1995) *FEBS Lett.* **368**, 311–314
Leamon, C. P., and Low, P. S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 5572–5576
Perez, F., Ledo, P. M., Karagogeos, D., Vincent, J. D., Prochiantz, A., and Ayala, J. (1994) *J. Mol. Endocrinol.* **8**, 1278–1287
Prochiantz, A., and Théodore, L. (1995) *BioEssays* **17**, 39–45
Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. (1990) *EMBO J.* **9**, 1503–1510
Sandvig, K., and van Deurs, B. (1994) *Trends Cell Biol.* **4**, 275–277
Théodore, L., Derossi, D., Chassaing, G., Llirbat, B., Kubes, M., Jordan, P., Chneiweiss, H., Godement, P., and Prochiantz, A. (1995) *J. Neurosci.* **15**, 7158–7167
Troy, C., Derossi, D., Prochiantz, A., Greene, L. A., and Shelanski, M. (1996a) *J. Neurosci.* **16**, 253–261
Troy, C., Stefanis, L., Prochiantz, A., Greene, L. A., and Shelanski, M. L. (1996b) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5635–5640
Wade, D., Boman, A., Wahlin, B., Drain, C. M., Andreu, D., Boman, H. G., and Merrifield, R. B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 4761–4765

[2] G. Rossi, M. Volovitch, and A. Prochiantz, unpublished observations.
[3] D. Derossi, M. Thieffry, and A. Prochiantz, unpublished results.
[4] J.-P. Berlose, D. Derossi, A. Brunissen, O. Convert, and G. Chassaing, manuscript in preparation.